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Investigations into Protein Synthesis in Protoplasts Isolated from Laminae of *Hordeum vulgare*

by

©

Peter S. Summers

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF Ph.D

IN

Plant Biochemistry

Department of Plant Science

EDMONTON, ALBERTA

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Investigations into Protein Synthesis in Protoplasts Isolated from Laminae of *Hordeum vulgare* submitted by Peter S. Summers in partial fulfilment of the requirements for the degree of Ph.D in Plant Biochemistry.

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Abstract

Protein synthesis is a complex metabolic process which is required for the continued viability of protoplasts. Attempts to maximize protoplast protein synthesis were made by examining the effect of various treatments on the incorporation of [^{35}S]-methionine into TCA-precipitable polypeptides by barley protoplasts. The highest rates of protein synthesis (7 fmoles $min^{-1}\mu g\ chl^{-1}$) were obtained for the protoplasts suspended in 700 mM sorbitol at pH 5.5. Hypotonically incubated protoplasts had reduced rates of protein synthesis and CO_2 fixation. The observed decrease in rate of protein synthesis was not related to increased ribonuclease activity or decreased mRNA content but may have been a function of the increased plasmalemma damage incurred by protoplasts suspended in buffers of low osmotic concentration. Osmotic concentration did not influence the type of polypeptides synthesized by the protoplasts. Survival of the barley protoplasts without nutrients was poor with more than 50% of the protoplasts lysing within 18 hours. The addition of spermine to protoplast suspensions only slightly improved the survival rate.

Rates of barley protoplast protein synthesis could not be significantly improved with additions of betaine, Ca^{2+}, K^+, Mg^{2+} or 1 or 10 μM concentrations of amines. Spermine, spermidine and gramine at 1 mM actually inhibited the incorporation of [^{35}S]-methionine into TCA-preciptable protein. This reduction in the incorporation of methionine into protein is probably due to decreased uptake of radiolabelled methionine since 1 mM spermine was shown to greatly inhibit the transport of methionine and α-aminoisobutryic acid across the protoplast plasmalemma. In addition, acetate, DCCD and pH 7.8 impaired methionine incorporation into protein probably by also interfering with membrane transport of radiolabelled amino acid. Chloramphenicol and cycloheximide did not significantly affect amino acid uptake. $CaCl_2$ actually improved protoplast uptake of α-aminoisobutryic acid by 100% but did not improve the incorporation of [^{35}S]-methionine into protein. Chloroplast protein synthesis was sensitive to treatment with chloramphenicol whereas cycloheximide specifically inhibited cytoplasmic protein synthesis. The synthesis of herbicide-binding protein was stimulated by light. Furthermore, light increased and diuron and atrazine retarded the turnover of the

herbicide-binding protein as has been previously reported for aquatic plant cells and isolated thylakoid membranes.

In comparison to severed leaves fewer proteins were radiolabelled by protoplasts and the small subunit of Rubisco was labelled to a lesser extent. On the other hand the radiolabelling of the herbicide-binding protein by severed leaves was much reduced in comparison to that by protoplasts. These differences in the proteins synthesized by protoplasts and severed leaves and the poor survival of Gramineae protoplasts remain issues for future research and concern.

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List of Abbreviations

AIB	α-aminoisobutryic acid
ADP	adenosine diphosphate
ATP	adenosine triphosphate
CAP	D-threo-chloramphenicol
CCCP	carbonyl cyanide *m*-chlorophenyl hydrazole
cDNA	complementary DNA
CHI	cycloheximide
chl	chlorophyll
CTP	cytosine triphosphate
DCCD	N,N'-dicyclohexyl carbodiimide
DNA	deoxyribonucleic acid
2,4-D	2,4-dichlorophenoxy acetic acid
EDTA	ethylenediaminetetraacetate
EGTA	ethyleneglycol-bis(β-aminoethyl ether)-N,N'tetraacetate
ESR	external standards ratio
FDA	fluorescein diacetate
HBP	herbicide-binding protein
IAA	indoleacetic acid
LSU	large subunit of Rubisco
Mes	2-(N-morpholino)ethanesulfonic acid
mRNA	messenger RNA
Mr	molecular weight relative to standards
Mw	molecular weight
NAA	α-napthyleneacetic acid
NS	not significant
oligo (dT)	oligothymidylic acid
PAGE	polyacrylamide gel electrophoresis
PAR	photosynthetically active radiation
PET	photosynthetic electron transport
poly(A)	polyadenylic acid
POPOP	1,4-bis[2-(5-phenyloxazoyl)]benzene
PPO	2,5-diphenyloxazole
pSSU	precursor small subunit of Rubisco
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
Rubisco	ribulose-1,5-bisphosphate carboxylase/oxygenase
SDS	sodium dodecyl sulfate
SE	standard error of the mean
SSU	small subunit of Rubisco
tricine	*N*-tris(hydroxymethyl)methylglycine
TRIS	tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid

I. Introduction

As research tools, protoplasts offer plant scientists numerous experimental advantages over intact plant tissue. For example, each protoplast may be uniformly exposed to chemical treatments thereby overcoming problems associated with concentration gradients and differential absorption by intact tissues. A suspension of protoplasts can be easily sampled and the protoplasts fractionated into their constituent components; the plasmalemma, nucleus, peroxisomes, mitochondria and chloroplasts. The study of metabolic pathways either in part or collectively with other pathways is facilitated by the use of protoplasts. Furthermore, protoplasts can be prepared from an extensive number of plant species and from various plant tissues. As such, the metabolism in different tissues and/or plants can be compared in parallel by use of protoplasts. However, if protoplasts are to be used as a plant system deemed amenable to experimental manipulation, it is important to determine the extent to which protoplast metabolism differs from that of intact tissue. Unfortunately, not all studies have shown the metabolic state of protoplasts to be identical to *in vivo* conditions.

Discrepancies in results obtained from protoplasts and intact tissue have been reported for lipid metabolism (252), protein synthesis (148,187), transport across the plasmalemma (236) and hydrolytic enzyme activity (126). Despite these anomalies a number of studies have emphasized the advantageous similarities between intact tissue and protoplasts (58,130,145,156). In instances where disparities between protoplasts and intact tissue have been reported, researchers have attributed poor protoplast quality to membrane damage (68) and/or high osmotic concentrations (187).

In addition to plant biochemical studies another major area of research for which protoplasts have been used extensively includes genetic research and the ultimate improvement of crop varieties (23). Plant protoplasts can be used as sources of genetic variability. This genetic variation can appear spontaneously or it can be induced through the use of mutagenic agents (237). Genetic diversity can also be increased through protoplast fusion of sexually incompatible species or the uptake by protoplasts of extraneous genetic material (237).

Protoplasts can then be screened directly for resistance or tolerance to pesticides, pathogenic toxins or environmental stress (23,237). The desirable protoplast variants can be regenerated into intact plants and the transformed plants incorporated into a plant breeding program. Leaf mesophyll cells have been the cell type of choice for the preparation of protoplasts. These protoplasts can be produced with relative ease and in large numbers. Unfortunately, mesophyll protoplasts of cereals have never been regenerated into an intact plant (243). The regeneration of a Gramineae species has only been possible through the use of protoplasts derived from embryo cultures (243,244). Protoplasts derived from embryo callus possess two disadvantages. Firstly, embryos are understandably difficult to isolate in large numbers. Furthermore, protoplasts derived from unorganized callus tissue are not as genetically stable as those derived from differentiated tissue (23,237,244). A major concern of researchers interested in the regeneration of plants from cells and protoplasts is the lack of biochemical and physiological knowledge available on the viability and morphogenesis of single plant cells and protoplasts as they regenerate into whole plants (237).

The purpose of this study was to contribute towards the understanding of protoplast metabolism keeping in mind two identified problems. One major problem has been the inability to regenerate certain mesophyll protoplasts into intact plants and the second problem has been the ever present concern that protoplast metabolism deviates from that of intact tissue. As protein synthesis is required for the continued viability, growth and subsequent differentiation of protoplasts into plants, this study has focused primarily upon this metabolic event.

Metabolism in protoplasts is reported to be affected by osmotic concentration (186,59). It was reasoned that if osmotic stress contributed to poor cereal protoplast viability and regeneration, then a study of the effects of osmotic concentration upon protoplast metabolism could answer some questions concerning poor cereal protoplast viability and the possible sources of metabolic differences between protoplasts and intact tissue. Therefore, protein synthesis, mRNA content, photosynthesis, chlorophyll content, protoplast intactness

and RNase activity were evaluated in barley protoplasts isolated and incubated in buffers of different osmotic concentrations.

Additional studies on protein synthesis in protoplasts were conducted using the osmotic concentration (700 mM sorbitol) which provided the best rate of protein synthesis. An attempt was made to improve protein synthetic rates by the addition of polyamines and mono- and di- valent cations. Both the addition of polyamines and cations are recommended for other protein synthesizing systems (77,136,185). The nature of the unexpected inhibition of protoplast protein synthesis by polyamines at concentrations previously recommended for oat protoplasts (60,109) was examined.

The response of protoplast protein synthesis towards inhibitors of protein synthesis and photosynthesis and the feasibility of using protoplasts as tools to study light regulation of protein synthesis and degradation was also evaluated.

II. Literature Review

A. Protein Synthesis in Plants

Protein synthesis in plants is accomplished by three systems which can be considered separated with respect to their spatial distribution yet somewhat 'integrated' with respect to function. The three systems include that of the cytoplasm, which translates nuclear encoded messages, and those of the chloroplast and mitochondrion. Although both the chloroplasts and the mitochondria possess their own DNA and RNA and are equipped to carry out processes of transcription and translation indicative of a high degree of autonomy, it is estimated that these organelles must obtain more than 90% of their polypeptides from the cytoplasm (51,127). Indeed, estimates based upon *in organello* protein synthesis show that the chloroplast may only synthesize 100 and the mitochondrion 20 of their own polypeptides (36,46,51,75,127). It is also evident that these organelles must rely upon the cytoplasm for the import of a substantial number of metabolites required to carry out processes of protein synthesis in addition to the many other organellar functions (51). However, as many metabolites are also transported from the organelles to the cytoplasm, a certain degree of collaboration between these cellular compartments is implied.

Protein synthesis in the cytoplasm and within these organelles follows analogous steps of initiation, elongation and termination. However, the means by which these steps are carried out display a number of important distinctions. A fundamental difference resides with the ribosomes. That is, the organellar ribosomes are prokaryotic in nature in that they are 70S and are composed of 50S and 30S subunits (51). In contrast, cytoplasmic ribosomes are 80S and are composed of 60S and 40S subunits (153). Both RNA and polypeptide components associated with these two ribosomal classes are also dissimilar.

In the cytoplasm and the chloroplast, decoding of mRNA into specific amino acid sequences follows the "Universal Genetic Code" (51,153). The mitochondrion, however, possesses many exceptions to this code (127) and has only 25 different tRNA species in

contrast to the 32 normally required by protein synthesizing systems (127). Chloroplast tRNA and amino-acyl tRNA synthetases are often interchangeable with bacterial equivalents but can rarely substitute for cytoplasmic components (172).

The initiating aminoacyl-tRNA which binds to the initiating mRNA AUG codon is formyl-methionyl-tRNA in the organelles (139). The cytoplasmic equivalent is an initiator specific non-formylated methionyl-tRNA (140). The elongation and initiation factor proteins in the cytoplasm and in the organelles are also distinct (150,153,127).

Cytoplasmic mRNA is often distinguished from ribosomal and transfer RNA by a tail of polyadenylic acid residues (poly(A)) which is post-transcriptionally added to the 3' end of the message (19). This feature permits a rather rapid method of separating cytoplasmic mRNA from the far more abundant rRNA and tRNA by affinity chromatography using a number of techniques including oligo-d(T) cellulose or poly(U) sepharose (19,185). However, chloroplastic and mitochondrial mRNAs do not contain extensive poly(A) tracts (260) which makes separation of organellar mRNA from total RNA far more difficult.

The processes of transcription and translation also differ in their response towards many inhibitors. At the transcriptional level cordycepin (polyadenylation inhibitor) and α-amanitin (RNA polymerase inhibitor) inhibit transcription specifically in eukaryotic systems. On the other hand, RNA polymerases from organelles (and all other prokaryotic systems) are sensitve to rifamycins (66).

Cycloheximide (CHI) is probably the most widely used inhibitor of 80S ribosomal protein synthesis. CHI reversibly binds to the 60S subunit thereby inhibiting the translocation of peptidyl tRNA to the ribosomal "P" site (179,188). In algae CHI specifically inhibited cytoplasmic protein synthesis at 0.1 μg/ml yet had no effect on *in vivo* chloroplast protein synthesis at levels of 1.0 μg/ml (195). Following a 2 h incubation, total protein synthesis was inhibited 68% by 5 μg/ml CHI in oat protoplasts (65) and 70% by 20 μg/ml CHI in tobacco protoplasts (94). Inhibition of protein synthesis in tobacco protoplasts (94) was not a consequence of retarded uptake of radiolabelled methionine. Protein synthesis in isolated

chloroplasts of *Euglena gracilis* (175), *Olisthodiscus luteus* (194), *Spinacea oleracea* (17) and *Pisum sativum* (215) was not inhibited over a period of one hour by concentrations of CHI up to 36 μg/ml. In each of these studies the researchers indicated that CHI was being taken up rapidly by the chloroplasts.

It is important that CHI be used in experiments of relatively short duration as CHI appears to have inhibitory effects on arginine synthesis, respiration, RNA polymerase and chloride uptake (51,66,179). All of these processes can, in turn, exert subsequent adverse effects upon protein synthesis.

The most commonly used inhibitor of protein synthesis directed by 70S ribosomes is D-threo-chloramphenicol (CAP). CAP binds to the 50S ribosomal subunit and prevents the action of peptidyl transferase (179). In algae CAP had no effect on cytoplasmic protein synthesis at a level as high as 100 μg/ml (195). However, CAP at 50 μg/ml can completely inhibit protein synthesis in isolated chloroplasts of *Euglena gracilis* (175), *Olisthodiscus luteus* (194) and *Pisum sativum* (215). For tobacco protoplasts, 200 μg/ml of CAP did not affect amino acid uptake (94,198) and this concentration could inhibit total protoplast protein synthesis by 30% over 2 h (94). Known secondary effects of CAP include inhibition of respiration, photophosphorylation and SO_4^{2-} uptake (51). Other commonly used inhibitors of prokaryote-like protein synthesis include lincomycin, streptomycin, spectomycin and erythromycin (66). Interestingly, erythromycin inhibits protein synthesis in chloroplasts but not does not affect protein synthesis in mitochondria of tobacco (235).

B. *In organello* Protein Synthesis

In organello protein synthesis was first described by Bamji and Jagendorf in 1966 (9). Early studies used crude chloroplast preparations and amino acid incorporation was very low. Using Percoll gradients, preparations of chloroplasts which are greater than 90% intact have been isolated from *Pisum sativum* (56), *Spinacea oleracea* (17), *Euglena gracilis* (74,175), *Chlamydomonas reinhardtii* (129) and *Olisthodiscus luteus* (194). These isolated chloroplasts

are very competent at synthesizing proteins from exogenously added radiolabelled amino acid. The highest rate of protein synthesis for isolated chloroplasts is that reported for *Euglena* at 280 fmoles leucine incorporated ug chl^{-1} h^{-1} (175). Chloroplasts isolated for *in organello* protein synthesis from Gramineae species have typically been either of rather poor quality as judged by their protein synthetic efficiency (69,76,171) or, alternatively, of good quality but only available in very small quantities (11,52,51).

Also, as previously mentioned, chloroplasts are not wholly autonomous. For a period of 30 to 60 min isolated chloroplasts can continue many of their normal activities including photosynthesis and protein synthesis using pools of previously imported cytoplasmic components. Protein synthesis by isolated chloroplasts has not been shown to proceed for periods longer than 60 min and often does not exceed 30 min (169,194). This limitation presumably arises due to a deficiency of products supplied by the cytoplasm, a reduced amount of organellar mRNA and/or decreased organello membrane intactness (74).

The metabolic inhibitors diuron and carbonyl cyanide m-chlorophenylhydrazone (CCCP) are often used in studies of chloroplast protein biosynthesis. Diuron is an inhibitor of photosynthetic electron transport (242) and CCCP is an uncoupler of photophosphorylation (103). Both compounds can, therefore, inhibit ATP synthesis and, as a consequence, should ultimately inhibit protein synthesis. In some reports both diuron and CCCP were shown to inhibit protein synthesis (14,69,74,129) while in other studies they had no effect (56,194,17). This apparent contradiction may be due to the differential uptake of the compounds or may reflect different endogenous levels of *in organello* ATP pools.

C. Cytoplasm-Chloroplast Association

Proteins

Many chloroplast proteins including ribulose-1,5-bisphosphate carboxylase (Rubisco) (14), ATP synthetase (251) and 70S ribosomal proteins (17,42) have subunits synthesized in

both the chloroplast and the cytoplasm. Cytoplasmically synthesized polypeptides destined for the chloroplast are generally produced as higher Mw precursors (41). The N-terminal sequence is then cleaved during post-translational, ATP-requiring transport across the chloroplast envelope (27,79). Indeed some proteins like plastocyanin are transported across two membranes to the inner side of the thylakoid membrane (80).

Protein transport has been demonstrated using *in vitro* synthesized precursors and isolated intact chloroplasts (80) yet little is known about the steps involved in the assembly of organello and cytoplasmic subunits within the chloroplast. A cytoplasmically-synthesized chloroplast protein which non-covalently binds the large subunit of Rubisco (LSU) has been implicated in the assembly of LSU with the small subunit of Rubisco (SSU) (92).

An intriguing problem for researchers has involved understanding intergenomic regulation of the synthesis of chloroplast proteins. The abundance of Rubisco has made it a popular model to use in the study of chloroplastic/cytoplasmic cooperation in polypeptide synthesis, transport and subunit assembly (51). Light can stimulate the synthesis of the precursor to the SSU (pSSU) in pea and *Lemna gibba* through increased levels of transcription (223,231). Light regulation of pSSU mRNA synthesis appears to be less stringent in barley (247), cucumber (248) and mungbean (238). Light also stimulates the accumulation of the chloroplast encoded LSU. For the LSU, light regulation appears to operate at the levels of chloroplast gene copy number, transcription and translation (101,207,240). Therefore, light can regulate the ultimate synthesis of both subunits but this does not preclude the presence of other control measures.

A number of researchers have attempted to answer questions on the coordination of protein synthetic activities between the cytoplasm and chloroplasts. Many of these studies have provided contradictory results. For example, the use of protein synthesis inhibitors has suggested the coordinated synthesis of the LSU and SSU of Rubisco (102,165,258). Other studies using 70S ribosome deficient rye (54) and protein synthesis inhibitors with soybean cells (10) have demonstrated differences in the levels of LSU and SSU which suggests the

synthesis of the pSSU and LSU is not tightly coupled. A more recent and more complete study using the *Chlamydomonas reinhardtii* ac-20 mutant also shows a lack of strict coordination between the synthesis of the large and small subunit of Rubisco. When this mutant is grown under specific nutritional conditions it lacks 70S ribosomes. Therefore, under these conditions the cells are unable to synthesize Rubisco LSU. Previous studies which examined this mutant concluded that the absence of LSU translation in the plastid resulted in a failure of pSSU translation in the cytoplasm (72,102,165,258). This finding along with other studies (152) supported a condition of tight-coupling between products synthesized in the cytoplasm and the chloroplast. More recently, however, it has been demonstrated that the *Chlamydomonas reinhardtii* ac-20 mutant possesses wild-type levels of pSSU mRNA, that this mRNA is associated with polysomes and ribonucleoproteins and that the failure to accumulate detectable levels of SSU is a consequence of the rapid proteolytic degradation of unassembled SSU in the chloroplast (211).

Non-protein Metabolites

In addition to the transport of polypeptides into the chloroplast there is a substantial volume of traffic across the chloroplast envelope involving carbohydrates, elemental ions and amino acids. Gases and most neutral compounds diffuse through the chloroplast envelope very rapidly (89). In contrast, the chloroplast envelope is impermeable to sucrose, sorbitol and mannitol (91,89). The transport of other five and six carbon sugars and maltose is very low (3 to 9 μmol mg chl^{-1} h^{-1}) (93,89,208).

Many early studies of amino acid transport suggested that leucine passed into the chloroplast by way of very slow diffusion (170). A more recent study which employed a lower and more physiologically representative concentration of leucine has shown that although leucine diffusion is slow, leucine uptake by the chloroplasts of pea is mediated by a carrier mechanism (135). The passive diffusion of other neutral and polar amino acids is slow but continues at rates sufficient for chloroplast protein synthesis (71,89,170). The hydrophobic

amino acids phenylalanine and isoleucine appear to partition into the membrane lipid and therefore can diffuse rapidly (71). Glutamate, aspartate, glutamine and asparagine are transported across the chloroplast envelope at rates of 20 to 100 μmoles mg chl^{-1} h^{-1} via the dicarboxylate transporter (89) and by a high affinity transport system at rates of 2 μmoles mg chl^{-1}h^{-1} (256).

The chloroplast envelope is relatively impermeable towards Ca^{2+}, K^+, H^+, Mg^{2+} and NH_4^+ but very permeable towards Cl^-, acetate, nitrite and nitrate (37,89,158). Although the chloroplast envelope does not allow the rapid permeation of Ca^{2+}, K^+ and Mg^{2+}, these ions are important to chloroplast metabolism and may accumulate to total (soluble plus bound) concentrations of 32, 107 and 72 mM, respectively (84). The uptake of these ions is believed to be balanced by proton extrusion (84).

A cytoplasmically-synthesized phosphate translocator of Mw 29,000 daltons has been identified and characterized in the chloroplast envelope (62). This protein facilitates the rapid exchange of chloroplast phosphate in the form of triosephosphate or 3-phosphoglycerate for inorganic phosphate at rates of 300 μmoles mg chl^{-1} h^{-1} (89). In the dark, ATP is transported into the chloroplast via an adenylate exchange transporter. In the light, ATP and NADPH equivalents are transported as dihydroxyacetone in exchange for 3-phosphoglycerate (89,200).

D. Protein Turnover

The rate at which a radioactively labelled amino acid is incorporated into protein is often taken as a measure of protein synthetic capacity. This measurement is only an indication of gross protein synthesis. Several components of gross protein synthesis include the rate of protein synthesis, the rate of protein degradation, the fraction of label being recycled and the rate of uptake and final specific activity of the labelled precursor in all of the cellular metabolic pools (34,35). A number of techniques have been used to separate rates of synthesis from rates of degradation. These methods are heavy water density labelling of protein (28), the determination of tritium in the α-carbon of amino acid protein after ^3H_2O

incubation (216), long term tritium labelling followed by determination of carbon-2-^{3}H in protein amino acid (100) and double isotope labelling of protein (34). The analysis of factors contributing to gross protein synthesis is difficult with whole tissue since exogenously added compounds are often taken up by cells at different rates. An ideal system for plant protein turnover studies would be a homogenous population of single cells with a cell membrane which is permeable to all precursors, inhibitors and indeed any other factors whose effect is to be studied by experimentation. Suspensions of aquatic plant species offer uniform, easy to manipulate systems with a minimum number of cells per plant for the study of protein synthesis and degradation. Protoplasts, callus and single cells may also be useful for these studies but they have rarely been used to study protein degradation.

Increased total protein degradation and decreased total protein synthesis has often been associated with tissue senescence (64,180) and conditions of stress (29,45,44). Since Rubisco makes up more than 50% of the soluble protein in a leaf (51) it is not surprising that 85% of the protein degradation in a senescing leaf is due to loss of this enzyme and that this protein degradation is correlated with decreased rates of CO_2 fixation (64,180). Also correlated with senescence are decreased chlorophyll content, decreased free α-amino nitrogen and increased free non-reducing sugars and proteolytic activity (64,180). Cycloheximide inhibits and kinetin retards these changes attributed to senescence (44,180).

It is not known how the plant selects proteins for degradation or why some proteins turn over more rapdily than others (29). One protein that has been identified as being rapidly synthesized and degraded is the chloroplast encoded herbicide-binding protein (HBP) (144,193,192,230). This protein is probably the QB protein involved in regulating electron transport between the Q protein of photosystem II and the plastoquinone pool (144,230). The protein is ubiquitous to higher plant species including the triazine-resistant biotypes (95,142,143,230). The synthesis of this protein appears to be regulated by light at both transcriptional and translational levels (47,254). The degradation of this protein *in vivo* (142,173) or in isolated thylakoids (122,259) is very rapid and is also a function of light

intensity. The degradation of the HBP in light is estimated to be 50 times faster than that of Rubisco or the light-harvesting chlorophyll protein (142). This degradation is retarded in the presence of diuron, atrazine or ADP (142,122,173,259). The mechanism responsible for the loss of the HBP does not appear to be ATP-dependent (142,173,259) and is not inhibited by the protease inhibitors antipapain (173), EDTA (259), phenylmethylsulfonyl fluoride (173) and chloroquine (259). However, the thioprotease inhibitors Cu^{2+}, Hg^{2+} and *p*-hydroxymercuribenzoic acid and the endoprotease inhibitor α_2-macroglobulin significantly reduced the loss of the HBP during light incubation of thylakoid membranes (259). As previously mentioned, the rate of HBP loss from thylakoid membranes accelerates if the light intensity at which the membranes are incubated is also increased. However, when thylakoid membranes are incubated under high light intensity in the presence of the terminal electron transport acceptor, ferricyanide, the degradation of the HBP is reduced (173). These observations, including the prevention of HBP loss by diuron or atrazine, suggests that HBP degradation is closely linked to photosystem II PET activity (6). The phenomenon of photoinhibition has characteristics which are closely related to those just described for HBP loss (6). Photoinhibition is the inhibition of photosystem II PET activity under high light intensities and/or high temperatures (6,161). The HBP is required for photosystem II PET and the loss of the HBP under high light intensities has been correlated with photoinhibition (122,173). It has been suggested that the HBP acts as a photooxidizable scavenger during maximal rates of PET (6,47,122).

In addition to the HBP the synthesis and degradation of two nuclear-encoded proteins is reported to be very rapid. Studies with NADPH:protochlorophyllide oxidoreductase (78) and a 17 kD thylakoid membrane protein (147) have shown the half-life for these proteins to be only 2 to 3 h. In addition, rates of synthesis and degradation of these proteins and their mRNA appear to be light-regulated (78,147).

E. Ribonucleic Acid Turnover

The transcription of mRNA is usually discussed concomittant with the regulation of protein synthesis. The degradation of RNA and its effect on protein synthesis and cell regulation is rarely considered. Plant RNases are mostly soluble or loosely bound to membranes and are associated with all plant cell components (53). Increases and decreases in total RNase activity have been noted for a number of plant development stages (53). However, no specific plant metabolic stage has been shown to possess characteristically high or low levels of RNase activity. The only uniform response of RNases is their increase during plant stress. Measureable increases in the rates of RNase activity can be detected within 1 to 2 h of wounding a plant (7,53). Diseased plants (25) and water stressed plants (43) or protoplasts (65,187,186) also exhibit increased levels of total RNase activity.

It is not known whether RNases control the turnover of any specific mRNA species. Many mRNAs have short half-lives (147,119) which suggests a continual breakdown of mRNA. In contrast, the period required for the turnover of rRNA appears to be many days (241).

F. Polyamines in Plants

The diamine putrescine and the polyamines spermidine and spermine occur ubiquitously in the bacteria, plant and animal kingdoms. A number of other polyamines including cadaverine and canavalmine are found in only some plant, bacteria or animal species (224).

In plants, putrescine is synthesized from ornithine via ornithine decarboxylase or by decarboxylation of arginine by arginine decarboxylase to agmatine and N-carbamoyl putrescine (220,224). The latter pathway is not present in animals. Spermine and spermidine are synthesized from putrescine by sequential transfers of amino groups from decarboxylated S-adenosylmethionine (218,220,224,233). The above pathways, including the synthesis of methionine, occur solely in the soluble cytoplasmic fraction (217). Diamines and polyamines

can be catabolized by diamine and polyamine oxidases, respectively. Polyamine oxidase has been located in the cell walls of oat leaves (112).

Many studies have attempted to understand the function of naturally occurring polyamines. The polyamines are highly charged compounds which, in theory, should bind strongly to the phosphate of phospholipids and nucleic acids. Polyamines do form stable complexes with DNA, tRNA and rRNA (233). A proposed function for these complexes is nucleic acid structure stabilization and gene expression (220,233). Polyamines could also be involved in regulating cytoplasmic pH (220). Polyamines exert both inhibitory and stimulatory effects upon many enzymes. Predominant among the enzymes positively modulated by polyamines are protein phosphorylases. One species of these enzymes phosphorylates and thus inactivates ornithine decarboxylase, an enzyme involved in polyamine biosynthesis (233). Many other enzyme reactions are reversibly inactivated by polyamines including RNase hydrolysis of RNA (110). However, these effects are believed to be due to non-specific cationic binding (220,233).

The polyamines as well as Ca^{2+}, have been reported to stabilize plant membranes and, hence, delay senescence (113,115,128,182). More recently, a report suggests that this apparent stabilization can be explained by the ability of polyamines and Ca^{2+} to reduce membrane fluidity (128,197,210). The rigidified membranes are in an "animated" state and are unable to continue physiological functions requiring a fluid membrane (12,182,197,227,228).

In all cell-free translation systems polyamines are known to stimulate protein synthesis and improve translational fidelity (1,77,153,233). A study using rat hepatoma cells exhibited a correlation between spermidine depletion and diminished polysome profiles (50). Similarly, a correlation between the presence of polyamines and ribosome assembly suggests that polyamines may stabilize ribosomal subunits (233). The positive benefits of polyamines towards eukaryotic protein synthesis may not hold true for prokaryotic systems. High concentrations of Mg^{2+} and spermidine can adversely affect protein synthesis as demonstrated by some studies using *E. coli* (233). In *Anacystis nidulans*, 150 μM putrescine completely

inhibited protein synthesis and caused ribosomal disaggregation (82). Protein synthesis by isolated thylakoid-bound ribosomes was also inhibited by 500 μM putrescine (13).

Experiments using intact plant cells have also revealed negative effects in response to exogenously supplied polyamines. Rose callus viability was reduced by the addition of 1 mM spermine, 1 mM spermidine or 10 mM putrescine and improved with 1 mM putrescine (159). A number of studies have noted trends in the titer of polyamines in tissue and the extent of cell division and differentiation occurring in the tissue. In general, the activities of enzymes involved in polyamine biosynthesis and polyamine content increased in tissue undergoing rapid growth and development (55,114,177,219). This observation contrasts with the rapid decline in polyamine content of tissue undergoing senescence (73,114,116,222). Exogenous applications of compounds which inhibit the activity of various enzymes involved in polyamine biosynthesis reduced the levels of polyamines in the tissues treated and also inhibited mitotic activity, cell division (114,249) and embryogenesis (55) but did not appear to affect cell elongation (249). Therefore, there appears to be a temporal correlation between polyamine titer in plant tissue and plant growth and development.

Polyamine content of various plant tissues can attain values as high as 500 nmoles/g FW (73,222,239). *Saintpaulia ionantha* petals can accumulate putrescine at a rate of 2200 nmoles g $FW^{-1}h^{-1}$ (8,181). It is, therefore, possible that after an exogenous application of a polyamine intact leaf tissue could attain at least four times the natural concentration of the polyamine in one hour. The uptake of polyamines by protoplasts, cells and callus may be even greater leading to even higher polyamine concentrations.

Polyamines, especially putrescine, also appear to accumulate under conditions of osmotic stress, potassium deficiency, acidity and salt stress (61). For each of the above conditions it is important to note that senescence did not accompany the induced stress. The increased levels of putrescine as a function of stress were paralleled by increases in arginine decarboxylase activity (264). The increased activity of this enzyme is probably due to its *de novo* synthesis (61,264). The mechanism which has been hypothesized to account for all the

above stresses is that of cation/anion imbalances in the intracellular spaces which could be alleviated by the accumulation of putrescine (61,224).

In conclusion, the roles of polyamines in plants have remained largely an enigma. Although many beneficial effects appear to be exerted by these compounds (55,114,177,219), it is clear that these compounds may also exert serious deleterious consequences upon many plant metabolic activities (12,13,159,182,197,227,228).

G. Use of Plant Protoplasts to Study Plant Cell Metabolism

Plant protoplasts are isolated plant cells lacking the semi-rigid cell wall composed of cellulose, hemicellulose, pectic polysaccharides and structural proteins. Protoplasts can be released from various tissues and plant calli with the use of fungal cellulases and pectinases (174,243). Suspensions of protoplasts can then be used for genetic transformations (23,237), plant pathological studies with viruses (117,160) and fungal toxins (206), the isolation of intact cellular components (68,154,166,168,189), studies of intracellular metabolism and transport of cellular metabolites (85,162,262,263), the uptake across the plasmalemma of various exogenously applied compounds (132), photosynthesis (164) and protein synthesis (205). Some notable uses of protoplasts have been for the identification of hormone-regulated proteins (104,148), the study of cell wall biogenesis (63,68) and the isolation of C_4 mesophyll cells (21,49). Broglie *et al.* (21) isolated mesophyll protoplasts from *Zea mays* and mesophyll and bundle sheath cells were then employed for an intense study of the molecular basis for the differential localization of enzyme activities. For both cell types a number of enzymes were analyzed for *in vivo* quanitity and specific activity. In addition, the mRNA coding for these enzymes in the two cell types was quantitated by Northern blot analysis and the amount of enzyme translated *in vitro* from poly(A) RNA isolated from these cell types was also quantitated. Such a study was possible because protoplast technology allows one to obtain purified, intact mesophyll cells (21). Edwards has used protoplasts for the isolation and evaluation of photosynthesis in many C_4 species (48,154,189). Recently, Edwards and Scott

(49) described the use of protoplasts to study photorespiration in C_3 and C_4 species. They were able to isolate chloroplasts, mitochondria and peroxisomes from both mesophyll and bundle sheath cells and subsequently study photorespiratory enzyme activity (49).

Plant Protoplasts Under Stress

Once plant protoplasts are released from their cell wall they enter a stressed state. The osmotic concentration and the media components are alien to the protoplast. In addition, the intercellular plasmodesmata have been severed and the plasmalemma is often damaged during isolation (68). Protoplasts also appear to undergo ultrastructural changes during the transition from cell to protoplast. These changes include increases in chloroplastic pseudocrystals, decreases in chloroplast and thylakoid volumes and a reduction in ribosome numbers (70,151). These changes appear to be largely reversed during the meristematic growth of tobacco cells into callus (70).

The degree of stress that a protoplast endures is difficult to assess. Although the ability of protoplasts to regenerate into plants has been used as such a measure (16), the regeneration of viable plants from protoplasts has been achieved for only a few species (81,114,225,243). Plant regeneration has never been accomplished from cereal leaf protoplasts (60,183,243). It is possible that cereals are either more sensitive to the protoplast isolating conditions and/or differentiated cereal laminae cells are genetically incapable of cell initiation (60,183).

Possible genetic or developmental factors which impair protoplast regeneration have not been examined to any great extent. Many phenomena which have been specifically noted for protoplasts and not for intact growing leaf tissue are also generally associated with plant senescence. These observations include chlorophyll loss (109), increased RNase activity (2,186) and decreased RNA and protein synthesis (2). A number of other cellular functions may differ between protoplasts and the leaves from which they are derived. Comparisons between leaves and protoplasts have been made for the light and dark reactions of

photosynthesis (108,107,155), lipid and fatty acid composition (252), membrane concentration (20) and metabolite movement across the plasmalemma (132,156).

Ribonuclease Activity

RNase activity of protoplasts increases dramatically with incubation time (67,126). This increase is probably due to *de novo* protein synthesis of RNases as this increase is prevented by cycloheximide (126,186). When protoplasts or leaf discs are incubated with a high concentration of osmoticum, RNase activity is significantly higher than if the same tissue was incubated in a buffer of low osmotic concentration (126). Farkas and his co-workers (126,186) have suggested that the hypertonic solutions used for protoplasts contribute to the increased RNase activity.

At physiologically high concentrations (10 mM), polyamines and dibasic amino acids were able to inhibit oat protoplast RNase activity by up to 50% (110). RNase activity did not increase in protoplasts treated with 1 mM spermine for 3 h (110) or 10 mM arginine for 18 h (2). The rate of chlorophyll loss in protoplasts was also retarded by polyamines, diamines and cycloheximide (109).

Macromolecule Biosynthesis

Protoplasts are able to incorporate radiolabelled amino acids into protein for a period of hours (59,65,94,205,265). In species which are amenable to regeneration from mesophyll protoplasts (*Solanaceae*, 81) protein synthetic activity can be extended to 30 or more hours if hormones are included in the culture medium (94,265). In these cases, plant regeneration usually begins within 12 h of culture (265). Hirai and Wildman (94) found that 2,4-D increased [^{35}S]-methionine uptake by tobacco protoplasts while kinetin improved protein synthesis by 30% (94). In contrast, protein synthesis in oat protoplasts was inhibited by kinetin and was unaffected by 2,4-D, IAA, NAA and gibberellic acid (65). Leucine incorporation by oat protoplasts was improved fourfold if lysine was included in the

incubation medium at levels of 10 or 100 mM (109). There are two reports that exposure of protoplasts to low levels of light (<300 lux) results in better rates of protein synthesis (59,94). It is interesting to compare the results of these biochemical studies with accepted tissue culture techniques used in the regeneration of plants from protoplasts. In general, a low light intensity is used during the first 6 to 14 days after plating. Auxins and cytokinins are included in most culture media for all species tested including Gramineae (243). Very little research has been carried out to relate the changes in metabolism of cultured cells and protoplasts to their growth during culture.

As discussed earlier, cycloheximide can inhibit increases in protoplast RNase activity suggesting that the increase in RNase activity is due to *de novo* synthesis of the enzyme. Something unique to the isolated protoplast must induce increased transcription of RNase and/or translation of RNase mRNA. The polypeptides synthesized from radiolabelled amino acids by tobacco protoplasts of varying ages have been compared following separation by polyacrylamide gel electrophoresis (PAGE). Although protein synthetic activity in freshly prepared protoplasts is higher than that found for older protoplasts, some proteins appear to be preferentially synthesized by the 'aged' protoplasts (57,58,59). Enhanced synthesis of a few proteins has also been noted for protoplasts or cells exposed to heat (149) and drought stress (39) and hormones (148). However, in the case of older tobacco protoplasts it is possible that the preferentially synthesized proteins are related to plant regeneration as opposed to protoplast stress or senescence (94).

Proteins synthesized in protoplasts have also been compared to those synthesized in calli or intact leaf tissue. When compared to leaves, the synthesis of some proteins in protoplasts has increased while the synthesis of the majority of proteins has decreased (57,58,59). The pSSU of Rubisco was synthesized in much smaller quantities in tobacco protoplasts than in cultured cells (58,59). The synthesis of LSU by protoplasts was also less, but the difference between protoplasts and leaves was not as dramatic. Also, cultured cells held in a hypertonic solution synthesized very little of either subunit (58). Subsequent studies

demonstrated that LSU mRNA, HBP mRNA and particularly pSSU mRNA were less abundant in protoplasts than in either hypertonic or isotonic cells (58) or the leaf tissue (57) from which the protoplasts and cells were derived. The use of a complementary DNA (cDNA) probe to pSSU mRNA has also shown that the pSSU mRNA is not present in protoplasts or white callus (245). Since the cytoplasmically synthesized pSSU is more adversely affected than the chloroplast-encoded proteins, 'osmotic stress' could be responsible, at least in part, for the inhibition of cytoplasmic transcription in protoplasts held in a hypertonic buffer.

Premecz and co-workers (187) attempted to look at protein synthesis in protoplasts without the added factor of transcription. They found that protoplasts derived from virus infected leaves and suspended in 400 or 700 mM mannitol synthesized virus protein equally well. However, the same authors state that histidine incorporation into protein was greater by protoplasts suspended in 400 as opposed to 700 mM mannitol even though the protoplasts suspended in 700 mM mannitol took up more histidine (187).

Uridine and thymidine incorporation into RNA and DNA, respectively, changes little with the 'age' of tobacco protoplasts (111). For at least 12 h uridine and thymidine can be linearly incorporated into protoplast nucleic acids (121,205,265) and incorporation of nucleotides into protoplast nucleic acids can be improved with the addition of polyamines and dibasic amino acids (111,220). Rates of protoplast nucleic acid synthesis have not been compared to *in vivo* rates of nucleic acid biosynthesis.

Photosynthesis

Protoplasts can be used directly to study light and dark reactions of photosynthesis (155,164,226) or intact chloroplasts obtained from protoplasts can be employed in analagous studies (48,189,226). Researchers are in agreement that rates of photosynthetic oxygen evolution and CO_2 fixation by protoplasts or chloroplasts derived from protoplasts are similar to those rates of intact leaf tissue (98,107,155,164,189). Indeed, protoplasts of *Arabidopsis*

thaliana had rates of CO_2 fixation which greatly exceeded those determined for the intact plant (226). Chloroplasts derived from protoplasts are also usually more photosynthetically efficient than mechanically isolated chloroplasts (167,189). However, some anomalous results concerning photosynthesis in protoplasts have been reported. Morris and Thain (157) found that the enzymes used in isolating protoplasts can inhibit photosynthetic activities. Protoplasts held in a hypertonic buffer had reduced rates of photosynthetic dark reactions compared to protoplasts held in an isotonic solution (107,155,157). This effect of osmoticum is not entirely reversible and is possibly due to excessive influx of solutes and/or the loss of metabolites and cofactors during the period of 'osmotic stress' (107).

Transport Across the Plasmalemma

A suspension of protoplasts is fairly homogenous with regard to cell type and size and is of known genetic composition (23). Additionally, protoplasts have a known ultrastructure which is less complicated and less heterogeneous than that of intact leaf tissue. All these factors make protoplasts ideal models for evaluating the transport of compounds across the plasmalemma. Protoplasts and tissues from which the protoplasts are obtained have similar kinetic and selectivity parameters for the uptake of a number of compounds including sugars, amino acids and ions (33,130,145,156,246,266). It has been shown that cell walls can selectively inactivate or bind some chemicals used in uptake studies (131,178), therefore, the use of protoplasts for such studies is advantageous.

The osmotic concentration of the protoplast buffer can reversibly affect ion uptake (157) but does not appear to alter membrane concentration (20). The membrane concentration of protoplasts derived from tobacco callus was not significantly different from plasmolyzed or unplasmolyzed tobacco callus cells (20).

Plant protoplasts can import a large variety of substances by a number of different methods. Plant viruses are efficiently taken up by protoplasts (117,174) probably by pinocytosis (160). The methods by which sugars, amino acids and ions are transported across

the plasmalemma are unclear but appear to involve carriers driven by reducing equivalents or a proton gradient (106,132,246). Very high rates of uptake have been reported for K^+, Cl^- and Ca^{2+} (in order of permeability) with reduced rates for $H_2PO_4^-$ and SO_4^{2-} (131,145). Various sugars (93,133,134,212) and the amino acids leucine and aminoisobutryic acid (AIB) can be taken up rapidly against a concentration gradient (198,202). The pesticides ethirimol (137), 2,4-D and atrazine (33) can diffuse into protoplasts very rapidly. In contrast, the protoplast plasmalemma is practically impermeable to mannitol and sorbitol with uptake rates ten times lower than that of $H_2PO_4^-$ (198).

The rate of uptake of all the above compounds is profoundly influenced by pH, temperature, the presence of competing compounds (132) and the concentration of the chemical being tested (132,133,134,246). The pH of the surrounding media affects the charge of the compound studied, the function of plasmalemma proteins, the plasmamembrane proton gradient (83,145,246) as well as the plasmalemma intactness (209). Influxes of sugars, amino acids and ions are greatest between pH 4 and 5 (83,130,131,134,212,246). However, an exception to this generalization is K^+ which permeates the plasmalemma more readily at pH 7 (130,145). Maximum uptake by protoplasts of K^+, Cl^- and sucrose was observed between 30 to 40°C (145,212). Various ionophores, membrane modifying agents and metabolic and ATPase inhibitors have been used with considerable success to study mechanisms of transport primarily because the plasmalemma is easily exposed to these chemicals and evaluation of the effects can be rapidly determined (132,134,146,201).

III. Materials and Methods

A. Materials

All reagents used were analytical grade and were obtained from Sigma or Fisher unless otherwise specified. All buffers, stock solutions and water were 0.22 μm filter-sterilized or autoclaved. All glassware, plasticware, cheesecloth and Miracloth (Calbiochem) were autoclaved. Methanol, ethanol and phenol were redistilled and the herbicides were recrystallized. Stock solutions of all chemical treatments were adjusted with HCl or KOH to the pH at which they were used.

B. Protoplasts

Seeds of *Hordeum vulgare* cv. Gateway were surface-sterilized in 5% (w/v) NaOCl for 10 min, thoroughly washed with distilled water and sown in shallow trays of vermiculite. The barley was grown for 6 d under continuous light (PAR of 100 $\mu E\ m^{-2}\ s^{-1}$) at 20°C. Apical 2 cm leaf segments of these plants were used for protoplast preparation. The harvested leaves were surface-sterilized by submerging them in 1% (w/v) NaOCl, 4% (w/v) Tween 20 for 5 min and then rinsed extensively with autoclaved water. After the leaves were washed all further steps in protoplast preparation and experimentation were performed aseptically. The abaxial epidermis was removed with fine forceps and approximately 15 g of leaves were floated on 15 ml of a 0.45 μm filter-sterilized digestion buffer of 400, 500, 600 or 700 mM sorbitol, 5 mM Mes-KOH (pH 5.5), 2% (w/v) Onozuka R-10 cellulase (Kinki Yakult, Tokyo), 0.1% (w/v) Y-23 pectolyase (Seishin Pharmaceutical, Tokyo) and 1% (w/v) bovine serum albumin (BSA) (2).

Digestion proceeded for 2 h in the dark at 30°C with gentle shaking. Protoplasts were filtered through three layers of cheesecloth and one layer of Miracloth then pelleted by centrifugation at 150*g* for 5 min at room temperature. After centrifugation the pellet was carefully resuspended in an incubation buffer of sorbitol and 5 mM Mes-KOH (pH 5.5). This

step was repeated four times. Protoplasts purified as above were free of cell wall debris although they did contain some free chloroplasts and broken protoplasts. Additional purification using sucrose/sorbitol (48) or sodium metrizoate/Ficoll (123) step gradient flotations did yield preparations free of chloroplasts. Unfortunately, the yield obtained by these methods did not provide sufficient protoplasts to conduct further analyses. Isolated protoplasts were counted using an haemocytometer and diluted to 1.6 x 10^6 protoplasts/ml. Protoplast intactness was evaluated with a Zeiss Fluorescent microscope and fluorescein diacetate (FDA) at 0.02% (w/v) was used as an indicator of membrane intactness (123). Chlorophyll concentration was determined by the method of Arnon (4). Protein was precipitated with 80% (v/v) acetone at -20°C overnight. The precipitate was pelleted by centrifugation at 10,000*g* for 10 min at 4°C. The pellet was dissolved in 2 N KOH and the concentration of protein was determined by the method of Bradford (18). Osmotic concentration of the buffers was measured with an Osmette A osmometer (Precision Systems, Newton, Mass.).

C. Protein Synthesis

Assays to determine the protein synthetic capacity of protoplasts were carried out in 15 ml Corex (Corning) tubes containing 60 or 200 µl final volumes. Protoplasts were suspended in incubation buffer containing the specific treatments as detailed in 'Results and Discussion' and a radioactive amino acid to yield a final concentration of 10^6 protoplasts per ml. The suspension of protoplasts was incubated in a 25°C shaking water bath set at 60 strokes per min. Light was provided by two 150 w flood lamps (PAR of 350 µE m^{-2} s^{-1}). The radioactive amino acids employed included L-[^{35}S]-methionine (originally >1100 Ci/mmol; 600 nM final concentration) and L-[3,4,5-^{3}H(N)]-leucine (>110 Ci/mmol; 600 nM final concentration) from New England Nuclear.

Two 10 µl aliquots were removed from each assay and spotted on to glass fiber discs (Whatman) at zero time and after the incubation period (usually 60 min). The discs were

processed as per Mans and Novelli (138) and counted in 10 ml of a toluene fluor containing 0.4% (w/v) 2,5-diphenyloxazole (PPO) and 0.01% (w/v) 1,4-bis[2-(5-phenyloxazoyl)]benzene (POPOP) (both from Amersham) in a Searle Isocap 300 liquid scintillation counter. Incorporation of radiolabelled amino acid into TCA-precipitable protein was corrected for zero-time values. Zero-time values were generally 4000 to 7000 cpm and 200 to 400 cpm per 10 μl of assay for [^{35}S]-methionine and [^{3}H]-leucine, respectively. Scintillation counting efficiency of ^{35}S on glass fiber discs was approximately 70%.

Turnover of the HBP was studied in protoplasts which were incubated under conditions slightly modified from those defined earlier. Protoplasts at 1.6 x10^6 protoplasts/ml were incubated with 1.2 μM [^{35}S]-methionine for 60 min under the previously described light and temperature conditions. Aliquots from this initial incubation containing 1.5 x 10^5 protoplasts were subjected to further incubations and treatments in order to examine the nature of HBP degradation in barley. To prevent the further synthesis of radiolabelled proteins by the protoplasts all treatments included 2.5 mM cold methionine or 100 μg/ml CAP and 25 μg/ml CHI. Final assay volumes were 150 μl and the final concentration of protoplasts (after addition of chemical treatments and cold methionine or CAP and CHI) was 10^4 protoplasts/ml. Untreated controls contained the same number of protoplasts as the treated samples. Samples were incubated either in the dark or under light intensities of 350 or 2500 μE m^{-2} s^{-1} all in a waterbath at 25°C. The higher light intensity was supplied by a 660 w quartz bulb. The incubations were terminated (including the untreated controls) by adding 200 μl of thylakoid isolation buffer (50 mM Tricine-KOH pH 7.8, 10 mM NaCl, 5 mM $MgCl_2$; 173). The samples were ground in a tissue grinder and the brei was centrifuged at 10,000*g* for 15 min at 4°C. The pellets were suspended in thylakoid isolation buffer without NaCl and recentrifuged. The final pellet was suspended in thylakoid isolation buffer without NaCl and an aliquot was removed for the determination of radioactivity in the thylakoid protein pellet. SDS-PAGE-solubilizing buffer was added to the samples and the total thylakoid protein sample from 1.5 x 10^5 protoplasts was loaded on to a polyacrylamide gel.

After each experiment protoplasts were streaked on Nutrient Broth agar plates. If bacterial contamination exceded one colony per 10^5 protoplasts, the assay results were discarded.

D. Polyacrylamide Gel Electrophoresis

Radioactive polypeptide samples were prepared in solubilizing buffer (50 mM Tris-HCl pH 7.5, 0.001% (w/v) bromophenol blue, 2% (w/v) sodium dodecyl sulfate (SDS), 20% (v/v) glycerol, 2% (w/v) dithiothreitol) and heated for 5 min at 90°C. Samples were then separated on 7.5-15% SDS-polyacrylamide linear gradient slab gels (26) using a discontinuous electrode buffer system (163). The resolving gel contained a 3 to 17% (w/v) gradient of sucrose for stabilization and was buffered with 424 mM Tris-HCl (pH 9.18). The 6% acrylamide stacking gel was buffered with 54 mM Tris-H_2SO_4 (pH 6.1). The upper reservoir buffer was 41 mM Tris-H_3BO_3 (pH 8.64) and 0.1% (w/v) SDS and the lower buffer was 424 mM Tris-HCl (pH 9.18). A Protean 32 cm gel apparatus (Bio-Rad) and a Buchler 3-1500 power supply were used. Gels were loaded with varying volumes of samples so that each lane contained equal cpms. Therefore, the total amount of protein loaded per well varied. Large quantities of protein often led to band broadening and distortion. SDS-PAGE molecular weight standards included [methyl-^{14}C]methylated β-lactoglobulin A (Mw 18,367), [methyl-^{14}C]methylated carbonic anhydrase (Mw 30,000), [methyl-^{14}C]methylated ovalbumin (Mw 46,000), [methyl-^{14}C]methylated albumin (bovine serum) (Mw 69,000) and [methyl-^{14}C]methylated phosphorylase B (Mw 97,400) all from New England Nuclear. Electrophoresis was carried out at 32 mamps for 9 h. Gels were stained for 8 to 10 h in 0.1% (w/v) Coomassie Brilliant Blue R-250, 25% (v/v) isopropanol, 10% (v/v) acetic acid and 0.1% (w/v) cupric acetate. After staining, gels were destained in 40% (v/v) methanol and 7% (v/v) acetic acid followed by soaking in destain solution plus 3% (w/v) glycerol for at least 1 h. Gels were then impregnated with ENlightening fluor (New England Nuclear) for 15 min (15) and dried between two sheets of cellophane (250). Dried gels were exposed to

Kodak XAR X-ray film at -80°C for varying lengths of time.

E. Immunoprecipitation of Radiolabelled Proteins

Antiserum to Rubisco holoenzyme of *Secale cereale* was a gift from Dr. J.D. Bewley, Dept. of Botany, University of Guelph. Dr. B. Grumbles, Dept. of Genetics, University of Alberta supplied the non-immune serum, the 10% suspension of formalin-fixed *Staphylococcus auerus* cells (Cowan 1 strain) and the protocol for immunoprecipitation.

Aliquots (20 to 100 µl) containing equal cpms of protoplast radiolabelled protein were ground in a tissue grinder and diluted with an equal volume of 40 mM Tris-HCl (pH 7.4), 2.5 mM Na_2-EDTA, 200 mM NaCl and 0.2% (w/v) Nonidet P-40. To the above, 10 µl of non-immune serum were added and after 15 min at 4°C the antigen-antibody complex was precipitated by the addition of 50 µl of *Staphylococcus aureus* cells. The cells were pelleted after 60 min at 4°C by centrifuging for 5 min at 12,000*g* and 4°C. The supernatant was added to 25 µl of Rubisco antiserum, incubated for 30 min at 20°C followed by 60 min at 4°C. The antigen-antibody complex was precipitated using 100 µl of *Staphylococcus aureus* cells during a 20 min incubation at 4°C. The precipitate was pelleted at 10,000*g*, 4°C for 3 min and washed at 4°C with the following buffers: 20 mM Tris-HCl pH 7.4, 1.0 mM Na_2-EDTA, 100 mM NaCl, 0.1% (w/v) Nonidet P-40; twice with 40 mM Tris-HCl pH 7.4, 1.0 mM Na_2-EDTA, 1.0 mM KCl, 1.2% (w/v) Nonidet P-40; once with 20 mM Tris-HCl pH 7.4, 10 mM Na_2-EDTA, 100 mM NaCl; and once with 20 mM Tris-HCl pH 7.4, 1.0 mM Na_2-EDTA, 10 mM NaCl. After the final wash, 100 µl of SDS-PAGE solubilizing buffer was added to the samples. The samples were heated for 5 min at 90°C, the *Staphylococcus aureus* cells were pelleted and the supernatant was loaded on to a polyacrylamide gel.

F. Photosynthesis Assay

The post-illumination CO_2 fixing capability of protoplasts was assayed in a buffer of 400, 500, 600 or 700 mM sorbitol, 50 mM Tricine-KOH (pH 8.0), 3 mM Na-ascorbate, 2 mM KH_2PO_4, 0.75 mM $MgCl_2$ (164,189). The above buffers were purged with N_2 gas and then used to dilute the protoplasts to a final concentration of 10^6 protoplasts/ml. The protoplasts were dark-adapted at 25°C for 2 min then incubated in light (same PAR as for protein synthesis) for 5 min at 37°C prior to the addition of $NaH^{14}CO_3$ (New England Nuclear) to a final concentration of 6 mM or 0.8 μCi/ml. Samples were incubated for an additional 10 min in the dark before adding an aliquot of the reaction mixture to an equal volume of acetic acid in a glass scintillation vial. After all the unincorporated $^{14}CO_2$ had been released the samples were counted in 10 ml of toluene fluor containing 33% (v/v) ethoxyethanol, 0.4% (w/v) PPO and 0.1% (w/v) POPOP. The samples channels ratio was used to convert cpms to dpms. All assays were repeated four times for each protoplast preparation.

G. Total Ribonuclease Activity

Total ribonuclease activity in soluble extracts was estimated as described by Altman *et al.* (2). Protoplast samples were ground in a tissue grinder, frozen in liquid N_2, thawed, and centrifuged at 10,000*g*, 4°C for 30 min. The supernatant containing the RNase was diluted so that the concentrations in the 500 μl assay were 50 mM Tris-HCl (pH 5.5), purified yeast RNA (Worthington Biochemical) at 0.75 mg/ml and protoplast supernatant equivalent to 2.0 x 10^5 protoplasts/ml. Assays were incubated at 37°C for 60 min and terminated by adding 500 μl of 2.5% (w/v) TCA, 0.3% (w/v) lanthanum nitrate. Samples were then stored at 4°C before being centrifuged at 10,000*g*, 4°C for 3 min. Nucleotides released from yeast RNA by ribonucleases were estimated from the increase in A_{260} readings. Each assay was duplicated and absorbances for controls containing TCA added before incubation were subtracted from each treatment value. Controls containing no yeast RNA substrate gave negligible values.

H. Quantitation of Rubisco mRNA

Total RNA extraction procedures were essentially as previously described (255). Total RNA was extracted from protoplasts 4 h after they had been isolated. The protoplast suspensions were frozen in liquid N_2 and ground to a fine powder with a mortar and pestle. The powder was suspended in 5 ml of RNA extraction buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM Na_2-EDTA, 40 μg/ml Proteinase K (Sigma,type XI) and 2% (w/v) SDS. After the suspension had shaken at room temperature for 4 h an aliquot was removed for chl determination. To this suspension, an equal volume of 80% (v/v) phenol was added followed by a similar volume of chloroform:isoamyl alcohol (49:1,v/v). After shaking for 30 min this mixture was centrifuged at 1000*g*, 4°C for 5 min. The upper aqueous phase was removed and extracted further with phenol:chloroform:isoamyl alcohol (50:49:1,v/v/v) until there was no flocculent protein interface. At this point only chloroform:isoamyl alcohol was added and and the samples were centrifuged at 1000*g*. The final upper aqueous phase was then added to 2.5 volumes of 95% ethanol to precipitate RNA overnight at -20°C. The RNA precipitate was centrifuged at 10,000*g*, 0°C for 10 min. The pellet was suspended in 2.5 ml of pellet wash buffer containing 3 M Na-acetate (pH 6.0) and 10 mM Na_2-EDTA then centrifuged at 10,000*g* for 10 min. This pellet was resuspended in 2.5 ml pellet wash buffer and centrifuged at 23,000*g* for 15 min at 0°C. The subsequent pellet was washed with 70% ethanol and finally dried using N_2(g). The final dry pellet was dissolved in 3 ml of 0.3 M NaCl and precipitated overnight at -20°C using 2.5 volumes of ethanol.

The precipitate was pelleted at 10,000*g*, washed once with 70% ethanol, dried and dissolved in 200 μl of water. Total RNA concentration was determined from A_{260} readings. One OD unit at 260 nm was taken to be equivalent to 40 μg/ml of RNA (136). All protoplast samples yielded approximately 1 μg total RNA per μg chl.

Total RNA at 0.5 μg/μl in SSPE buffer (180 mM NaCl, 10 mM NaH_2PO_4, pH 7.7, 0.1 mM Na_2-EDTA) and 6% (v/v) formaldehyde was denatured at 65°C for 2 min (119). Pre-hybridization, hybridization and stringency wash procedures are as described in the

Amersham Nylon Membrane Manual. Denatured RNA was diluted using 2XSSPE and 50 μl aliquots of the serial dilutions were transferred to a pre-wetted Hybond-N-Nylon membrane (Amersham) using a dot-blot manifold (Tyler Research, North Carolina). The aliquots contained 0.5, 1.0, 2.0, 5.0, 10.0 and 25.0 μg of total RNA. RNA was fixed to the membranes by exposing the membrane to an ultraviolet transilluminator for 5 min. The membrane was then pre-hybridized in a plastic bag for 8 h at 42°C with 25 ml of hybridization buffer containing 20XSSPE, 50% (v/v) formamide, 0.1% (w/v) each of BSA, Ficoll and polyvinyl pyrollidone, 50% (w/v) dextran sulphate and 20 μg/ml of calf-thymus DNA as a carrier DNA.

cDNA plasmids, *p*JZA4 (spinach LSU) and *p*BT (maize SSU) were a generous gift of K. Ko and N. Straus, Dept. of Botany, University of Toronto (120). Nick translation procedures were as described in the New England Nuclear Nick Translation Manual. Plasmids were nick-translated using the New England Nuclear ^{32}P-CTP nick translation system. The 25 μl reaction contained Tris-HCl, pH 7.4, $MgCl_2$, BSA, dATP, dGTP, dTTP, 1.2 Units of DNase 1, 50 μCi (100 nmoles) [α-^{32}P]-dCTP and 0.5 μg of denatured plasmid dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM Na_2-EDTA). The reaction was carried out at 12°C for 2 h and stopped by the addition of 5 volumes of 1% (w/v) SDS, 10 mM Na_2-EDTA, 100 mM NaCl and 50 μg/ml of salmon sperm carrier DNA. Nucleic acids were precipitated with ethanol at -20°C for 3 h. The precipitate was centrifuged at 10,000*g*, 0°C for 30 min. The unincorporated ^{32}P-dCTP in the supernatant was discarded and the pellet was washed with 70% ethanol. The final pellet was dissolved in STE buffer (TE buffer with 100 mM NaCl) and an aliquot was spotted on DE-81 paper (Whatman) and Cherekov radiation was determined by using the ^{3}H channel in the liquid scintillation counter. The hot probe contained at least 5 x 10^7 cpm/μg of added cDNA. To 25 ml of hybridization buffer, 1 x 10^7 cpm of radiolabelled probe was added and hybridization was conducted at 42°C for 48 h. The membrane was then washed twice with 50 ml 5XSSPE at 42°C for 15 min, once with 1XSSPE, 0.1% (w/v) SDS at 42°C for 30 min and once with 0.1XSSPE, 0.1% (w/v) SDS at

20°C for 15 min.

The membrane was then wrapped in Stretch n Seal (Imperial Oil) and exposed to Kodak XAR film at -80°C in a metal cassette containing two Coronex intensifying screens (Dupont). Control hybridzations performed against protoplast total RNA using control *p*BR322 plasmids as the labelled probes gave no hybridization signals.

I. Silicone Oil Centrifugation

Protoplasts were incubated at 1 x 10^6 protoplasts/ml under the same temperature and light conditions as described for protein synthesis. Radiolabelled methionine was used at the same concentration as in the protein synthesis studies (600 nM) but the specific activity was 60 times less (18 μCi/mmole; 11 μCi/ml). The non-metabolizable amino acid (213,246) [3-^{14}C] α-aminoisobutryic acid (New England Nuclear) was present at 82 mM (148 μCi/ml). For the determination of protoplast volume D-[U-^{14}C]-sorbitol (New England Nuclear) at 2.5 μCi/μl and ^3H_2O (New England Nuclear) at 0.04 μCi/μl were used.

The amount of radiolabel taken up by protoplasts over time was assayed by silicone oil centrifugation as described by Heldt (90). Four-hundred μl microfuge tubes were prepared with 75 μl of 2 N KOH followed by 100 μl of silicone oil (p=1.06; 1:17, 556:550 oils from Dow Corning). It was empirically determined that intact protoplasts could be pelleted through this density of silicone oil in 20 s using the Beckman B microfuge at full speed. No disrupted chloroplast membranes and a minimal number of intact chloroplasts passed through the silicone oil layer.

For each time period (including the determinations of protoplast volume) a 100 μl aliquot of the incubating sample was applied to the silicone oil in the microfuge tube, the tube was microfuged for 20 s, an aliquot of the supernatant was removed to verify the concentration of radiolabel and the tube frozen in liquid N_2. The tube was then cut in half, through the silicone oil layer, and the pellet resuspended in fluor. Both the aliquot of supernatant and the pellet were counted with 10 ml of toluene-ethoxyethanol-PPO-POPOP

fluor. The counting efficiency was determined by using an external standards ratio (ESR) and similarly quenched standards. ESR was selected so that the efficiencies of ^{3}H and ^{14}C or ^{35}S in the dual-labelled samples could be determined. Glass scintillation vials were used as toluene becomes absorbed to plastic vials and leads to erroneous ESR values (97).

The protoplast volume was determined as described by Heldt (90). Protoplasts were incubated simultaneously with $^{3}H_2O$ and [^{14}C]-sorbitol for 5 min before centrifugation. $^{3}H_2O$ freely permeates the protoplast and chloroplast membranes. In contrast, sorbitol does not permeate the plasmalemma or the chloroplast inner envelope and is used to correct for the amount of $^{3}H_2O$ and radiolabelled amino acid that adheres to membrane materials or is trapped inside among broken membrane material (90). The sorbitol permeable space was, on average, 8% of the total protoplast volume. The protoplast volume averaged 1.6×10^{-4} μl/protoplast. For each protoplast preparation the protoplast volume and the sorbitol impermeable space were determined and used in the calculation of amino acid concentration in the protoplasts.

IV. Results and Discussion

A. General Effects of Osmotic Concentration on Barley Protoplast Metabolism

In this study, protoplasts were subjected to osmotic concentrations of 415, 525, 636 and 738 mosmals l^{-1} (approximately -13 to -25 bars) by using sorbitol concentrations of 400, 500, 600 and 700 mM. These concentrations were chosen since they were within the range suggested by Okuno and Furusawa (174) as being suitable for barley protoplasts. Furthermore, the literature on protoplast osmotic stress has compared tobacco and oat protoplasts incubated in 400 and 700 mM mannitol (67,186,187). To minimize shock to the protoplasts they were maintained in media of the same osmotic concentration during digestion, isolation and incubation. In the following discussion the protoplasts will be identified by the millimolar concentration of sorbitol in which they were suspended.

Chlorophyll Content and Protoplast Intactness

On a per protoplast basis '400' and '500' protoplasts contained significantly greater amounts of chl than '700' protoplasts (Table I). Figure 1a illustrates a '400' protoplast preparation under the light microscope. The preparation has noticeably more intact, free chloroplasts than preparations containing other protoplast 'types' (Fig. 1b, 1c, 1d). The higher chl content associated with the '400' protoplasts (Table 1) probably reflects the greater number of free chloroplasts found in the '400' protoplast preparation. In turn, the free chloroplasts are an indication that this preparation has been subjected to more extensive protoplast lysis during isolation. The light-micrographs (Fig. 1) show '400' and '500' protoplasts to be larger than the '700' protoplasts, suggesting that the '400' and '500' protoplasts are turgid and swollen. The '700' protoplasts, in contrast, appear to be slightly plasmolyzed.

Previous studies noted that barley protoplasts survived better at higher (>500 mM) concentrations of osmoticum (174). It has also been reported that protoplasts of bean,

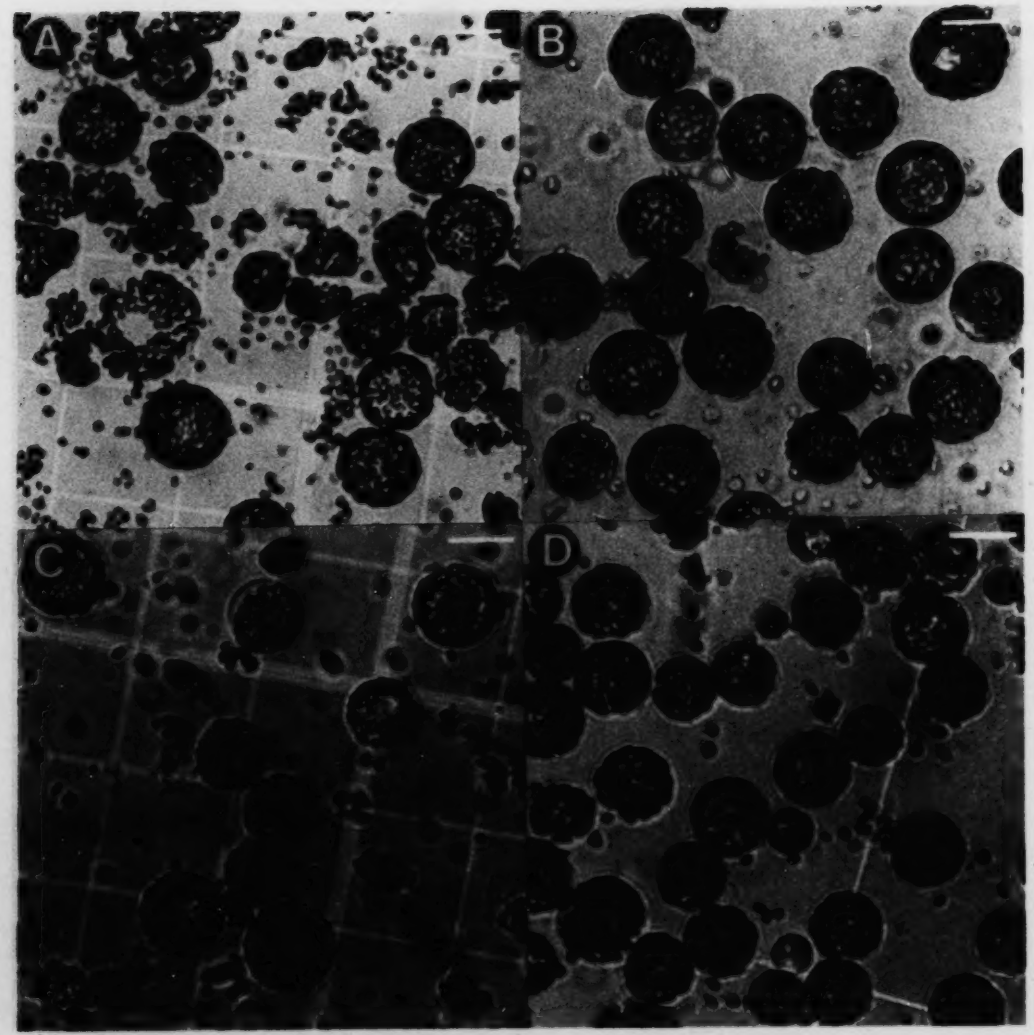
Table I. Characterization of protoplasts isolated and held in buffers of different osmotic concentrations. Aliquots of protoplasts suspended in 400, 500, 600 or 700 mM sorbitol buffers for 2 h after isolation were used to assay for RNase activity and chl. Protoplasts were deemed intact if they fluoresced in the presence of FDA. Values for intactness are the number of fluorescing protoplasts as a percentage of total discernible protoplasts. Intactness was measured 3 h after isolation and CO_2 fixation was measured 5 h after protoplast isolation.

Sorbitol Concentration (mM)	RNase Activity (A_{260} units per 10^5 protoplasts)	Chlorophyll (μg per 10^5 protoplasts)	CO_2 fixation (μmole CO_2 mg chl^{-1})	CO_2 fixation (nmole CO_2 per 10^5 protoplasts)	Intactness determined using FDA (%)
400	4.41	12.0	2.6	31	53
500	3.91	12.6	3.5	44	79
600	3.81	9.7	3.9	38	88
700	3.69	8.3	4.4	36	82
LSD(5%)	N.S.	2.6	0.8	7	N.S.
Number of Replicates	6	5	5	5	4

peanut, soybean, grape (22,118,209), oat (67,266), rapeseed (225) and spinach (167) rupture at osmotic concentrations equivalent to less than 500 mM sorbitol. Interestingly, light microscopic observations of hypertonically maintained (300 mM mannitol) spinach protoplasts revealed protoplasts which appeared to be intact. However, these protoplasts displayed aberrant permeability characteristics (167). The slightly plasmolyzed '700' (Fig. 1d) barley protoplasts should not be considered as 'abnormal'. When a cell, which is not a sphere, is converted to a sphere (e.g. a protoplast) the surface area of membrane required to encircle the same cell volume is reduced. Therefore, protoplasts derived from cells possess more plasmalemma than they require. The 'extra' membrane will appear convoluted (203). Since '700' protoplast preparations contain less chl (Table I) and fewer free chloroplasts and broken protoplast material (Fig. 1d), the plasmolyzed state of the '700' protoplasts would appear to be more resistant to breakage during isolation.

The definition of protoplast viability is rather vague and the use of fluorescein diacetate (FDA) as an indicator of protoplast viability has recently been questioned (16,167).

Figure 1. Light-micrographs of protoplasts digested and isolated in 400(a), 500(b), 600(c) or 700(d) mM sorbitol buffers. Protoplasts were prepared from 6 d leaves of barley. Bars represent 25 μm.


A
B
C
D

The non-polar FDA must freely permeate the plasmalemma before being cleaved to the fluorescing, polar, non-permeating fluorescein (123). Protoplast treatments which inhibit the uptake of FDA yet do not necessarily affect the internal viability of the protoplast may give a deceptive negative viability result with FDA. Indeed, a positive result with FDA may only indicate that the protoplast plasmalemma is intact and that the cytoplasm contains sufficient esterase activity to cleave the ester linkage between fluorescein and diacetate. It has been suggested that the regeneration of protoplasts into whole plants is the only conclusive test for protoplast viability (16). Despite these concerns, FDA can be used as a convenient indicator of protoplast membrane intactness.

Protoplasts held at different osmotic concentrations for 3 h after isolation did not possess any significant differences in intactness as determined by their ability to retain fluorescein (Table I). This lack of a difference could be attributed to the considerable variability inherent to different isolations of protoplasts. The quality of starting leaf material (108,176,184) and the isolation techniques (16,203) can significantly affect the quality of the final protoplast preparation. For example, preliminary results with barley protoplasts showed that a 30 s 70% (v/v) ethanol surface sterilization and/or wilting of leaf material led to very poor quality protoplasts. Both of these steps are recommended for the preparation of protoplasts from other species (2,68,203,243).

FDA was also used to determine the membrane intactness of '500' and '700' protoplasts incubated in the absence and the presence of 1 mM spermine for a 54 h period (Table II, Fig. 2). The survival of protoplasts in all incubation media decreased dramatically with time of incubation (Fig. 2). After 30 h of incubation only 21% of the protoplasts remained intact (Table II). By comparison, oat protoplasts incubated with hormones and a carbon source for 18 h had a survival rate of between 0 and 74% (2,67). In the experiment reported in this study nutrients were not included in the media and therefore the long-term survival of the barley protoplasts was not expected. The survival of the '700' protoplasts was marginally better than that found for the '500' protoplasts (Table II, Fig. 2). Although this

Table II. Protoplast intactness as affected by length of incubation, osmotic concentration and presence of spermine. Protoplasts were isolated in 500 and 700 mM sorbitol buffer and diluted to 2.0 x 10^4 protoplasts/ml with or without 1 mM spermine. Protoplasts were incubated in the dark, at 22°C, without shaking. At the indicated times after dilution, an aliquot was removed and added to an equal volume of 0.04% (w/v) FDA in sorbitol buffer. The number of fluorescing (intact) protoplasts were determined using a haemocytometer. Values are the number of intact protoplasts expressed as a percentage of the number of discernable protoplasts at time 0 (2.0 x 10^4 protoplasts/ml). Data are means of 3 independent replicates.

Sorbitol Concentration (mM)	Treatment	Incubation (h) 0	6	18	30	54
500	Untreated	95	58	23	12	3
	Spermine, 1 mM	92	69	39	17	8
700	Untreated	98	68	35	16	4
	Spermine, 1 mM	93	72	53	40	14
	Means	94	67	37	21	8
		a	b	c	d	e

Means followed by different letters are significantly different at the 5% level as determined by LSD. All interaction means are not significantly different at the 5% level. Spermine treated and untreated means are 50 and 41% respectively and at the 5% level they are significantly different. The means for the '500' and '700' protoplasts are 42 and 49% respectively and at the 5% level are significantly different.

finding is not consistent with the estimates of protoplast intactness based upon FDA measurements presented in Table I, this assessment does agree with the interpretation based upon the chl data in Table I. That is, reduced protoplast viability as judged by a raised chl content is greater in the '500' than in the '700' preparation.

In general, the survival of '500' and '700' protoplasts was improved by 9% when 1 mM spermine was included in the incubation buffer (Table II). The addition of 10 mM arginine only slightly improved the rate at which oat protoplasts survived (2,67). The addition of 1 or 10 mM putrescine, cadaverine, spermidine or spermine to oat protoplasts provided survival rates not significantly better than the untreated control (2). In the present study the percentage of fluorescing protoplasts relative to total discernible protoplasts for each

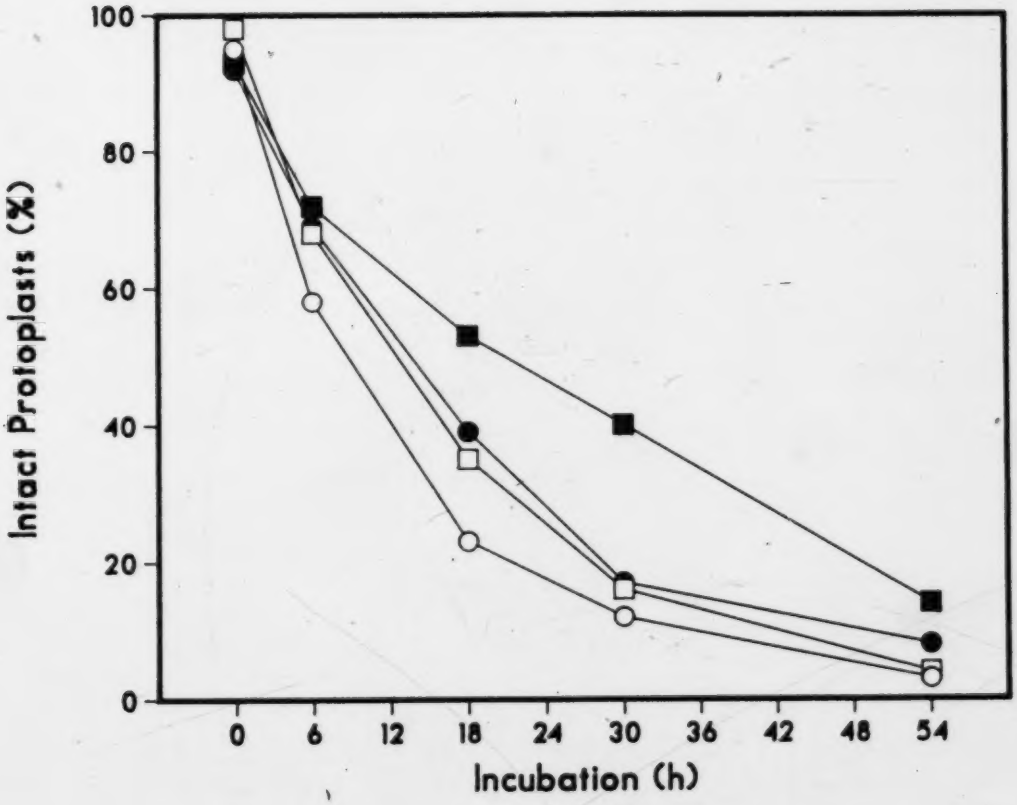


Figure 2. Protoplast intactness as affected by time of incubation, osmotic potential and the presence of spermine. Graphic illustration of the data in Table II. '500' protoplasts (○ , ●) and '700' protoplasts (□ , ■) in the absence (open symbols) or presence (closed symbols) of 1 mM spermine.

observation was constant. Spermine, therefore, does not appear to directly affect the uptake of FDA by the protoplasts.

Photosynthesis

On a per protoplast basis '700' protoplasts (5 h after isolation) appeared to be no more effective at fixing CO_2 than the '400' protoplasts (Table I). However, on a chl basis the '700' protoplasts had photosynthetic rates exceeding the '400' and '500' protoplasts (Table I). This difference can be attributed to the reduced number of free chloroplasts in the '700' protoplast preparation as compared to the '400' protoplast preparation. These free chloroplasts undoubtedly contribute towards the estimated amount of $^{14}CO_2$ fixed by the '400' protoplast preparation. Free chloroplasts in the '500' protoplast preparation also contribute to the quantity of $^{14}CO_2$ fixed. However, the extent of CO_2 fixation by the '500' protoplasts, expressed on a chl or protoplast basis, is significantly better than that of the '400' protoplasts (Table I). The values reported here for '700' protoplasts are 20% of those previously reported for barley protoplasts (189,214).

Kaiser *et al.* (107,108) conducted a number of experiments designed to assess the effects of osmotic stress on photosynthesis in spinach chloroplasts and protoplasts. An increase in the inhibition of protoplast photosynthesis was observed as the osmotic concentration of the protoplast suspending buffer was increased (108). This contradicts the results of the present study. Kaiser *et al.* (108) attributed reduced photosynthetic rates in hypertonically held protoplasts to soluble enzyme and metabolite leakage from the protoplasts. It is not known whether any of the barley protoplasts used in this study were subject to metabolite loss.

Ribonuclease Activity

Increased RNase activity has been associated with leaf senescence (53,109,113) and decreased protoplast vigour (67) including protoplasts under osmotic stress (126,186).

Therefore, since RNase activity has been associated with the quality of protoplasts from other species, the RNase activity in barley protoplasts subjected to different osmotic concentrations was examined. Protoplasts in suspensions will rupture during incubation (see Table II) releasing RNases into the supernatant (2,67). In previous studies the RNase activity increased in intact protoplasts as well as in aliquots containing the broken and intact protoplasts (67). In order to quantitate the RNase activity in the intact protoplasts the authors elected to re-isolate the protoplasts from the incubation media and then dilute the protoplasts to a known concentration (67). In the present study RNase activity in soluble extracts of samples containing intact protoplasts as well as suspending buffer was determined and no attempt was made to re-isolate the intact protoplasts.

The soluble extracts from preparations containing equal numbers of protoplasts suspended in buffers of different osmotic concentration for 2 h after isolation did not contain any significant differences in RNase activity (Table I). Over a 30 h incubation period '700' protoplasts contained slightly less RNase activity than '500' protoplasts (Table III). After 30 h of incubation the RNase activity in all the protoplast suspensions had actually decreased by 22% (Table III). A third inference drawn from Table III is that 1 mM spermine had no significant effect on the expression of RNase activity.

The conclusions described in the preceeding paragraph contradict those found for work carried out on tobacco and oat protoplasts (2,67,126,186). After isolation, RNase activity in tobacco (126) and oat (67) protoplasts is reported to increase for 12 and 24 h, respectively. The apparent increase in barley protoplast RNase activity after 6 h of incubation (Table III) was not significant. The increase in RNase activity in oat protoplasts was attributed to stresses inherent to the isolated protoplast (67). Premecz *et al.* (186) found that after 24 h of incubation tobacco protoplasts suspended in 700 mM mannitol had 35% more RNase activity than protoplasts suspended in 500 mM mannitol. They hypothesized that the increase in RNase activity in tobacco protoplasts was induced by osmotic stress (126). However, barley protoplasts suspended in a buffer of a higher osmotic concentration actually

Table III. RNase activity in suspensions of protoplasts as affected by length of incubation, osmotic concentration and presence of spermine. See Table II for experimental procedure. Aliquots were removed at the indicated times and frozen in liquid N_2. RNase activity was determined as detailed in 'Materials and Methods'. Values are means of 3 independent replicates expressed as A_{260} units per 10^5 protoplasts. Protoplast suspensions were diluted tenfold and therefore for the RNase activity assay the concentration of spermine in the treated samples was 0.1 mM.

Sorbitol Concentration (mM)	Treatment	Incubation (h) 0	6	18	30
500	Untreated	3.6	4.0	3.6	2.8
	Spermine, 1 mM	3.5	3.8	3.0	2.6
700	Untreated	3.0	3.5	3.0	3.0
	Spermine, 1 mM	2.7	3.1	2.9	2.5
	Means	3.2	3.6	3.1	2.5
		ab	a	b	c

Means followed by the same letter are not significantly different at the 5% level as determined by LSD. The means for '500' and '700' protoplasts are 3.4 and 2.8 A_{260} units, respectively, and at the 5% level are not significantly different. Spermine treatment was not significantly different from the untreated protoplasts at the 5% level. All interactions were not significant at the 5% level.

had less RNase activity (Table III). The increase in tobacco protoplast RNase activity was inhibited by CHI (126, 186). Therefore, the observed increases in protoplast RNase activity with time of incubation were attributed to *de novo* synthesis of the enzyme (126). Interestingly, the same authors (187) found total protein synthetic activity in protoplasts suspended in 500 mM mannitol to exceed that found for protoplasts suspended in 700 mM mannitol. Barley protoplasts also exhibit a general relationship between total protein synthesis and RNase activity. Barley differs somewhat in that the '700' protoplasts have a greater apparent capacity for protein synthesis (see 'Protein Synthesis Activity of Barley Protoplasts') and yet have reduced RNase activity (Table III) compared to '500' protoplasts.

Several reports have examined the ability of polyamines and dibasic amino acids to reduce increases in RNase activity in protoplasts (2,109,220). It is hypothesized that polyamines reduce RNase activity in protoplasts by 1) acting directly on the RNase hydrolysis

of the nucleic acids. It is known that spermine will bind to phosphate groups in nucleic acids and therefore is able to make the nucleic acid less susceptible to hydrolysis (233,110). 2) inhibiting the increase in enzyme levels. Kaur-Sawhney *et al.* (110) attempted to test the second hypothesis by removing the spermine from the protoplast suspension before assaying for RNase activity. However, the authors do not give any indication as to whether their dialysis procedure actually did remove spermine from the protoplast extract. Unlike the results for oat leaves and protoplasts (109,110,113), the addition of 1 mM spermine to barley protoplasts did not prevent the increase in RNase activity (Table III).

In vitro experiments have shown spermine to have a profound negative effect on the hydrolysis of RNA (110). The direct effect of polyamines and other RNase inhibitors on barley RNase activity was investigated. RNase activity of soluble extracts of barley protoplasts was assayed in the presence of polyamines, EGTA and heparin (Table IV). The indoleamine, gramine, and the diamines, cadaverine and putrescine, had no affect on *in vitro* barley RNase activity at 0.1 and 1 mM (Table IV). Spermidine at 1 mM and spermine at 0.1 and 1 mM were inhibitory with 1 mM spermine inhibiting RNase activity by 45%. The inhibition of RNase activity by the amines appears to increase with the degree of amination of these compounds. This observation supports the theory that RNA hydrolysis by RNase is inhibited through the binding of amine groups to nucleic acid phosphate groups (233). The degree of inhibition by polyamines found in this study (Table IV) is in agreement with that previously reported (110). EGTA binds Ca^{2+} with a high degree of affinity and therefore should inhibit many nucleases which require Ca^{2+} as a cofactor (136,124,261). However, the barley RNase was not inhibited by 10 mM EGTA. Heparin, a polysulfonated saccharide with many physiological functions in biological systems (31), is also reported to inhibit RNase activity (185). Heparin, at 2 and 10 mg/ml, inhibited barley RNase activity by 45 and 65%, respectively.

Table IV. Effect of amines, EGTA and heparin on *in vitro* RNase activity. Soluble extracts of barley protoplasts were incubated with yeast RNA for 60 min at 37°C in the presence of the indicated treatments. Values are means of 3 independent replicates expressed as percentages of the untreated control. Untreated control activity for soluble extracts from 10^5 protoplasts was 5.44 A_{260} units.

Treatment	Concentration (mM)	Percent of Control
Gramine	0.1	101.3a
	1.0	96.8ab
Cadaverine	0.1	98.9a
	1.0	98.8a
Putrescine	0.1	97.5ab
	1.0	89.0abc
Spermidine	0.1	94.3ab
	1.0	80.1c
Spermine	0.1	86.0bc
	1.0	55.1d
EGTA	10.0	91.9abc
Heparin	2.0†	55.5d
	10.0†	35.3e

† (mg/ml)
All means followed by the same letters are not significantly different at the 5% level as determined by LSD.

B. Protein Synthetic Activity of Barley Protoplasts

Protein synthesis is a fundamental requirement of any cell for growth and development. In the case of protoplasts, protein synthesis is essential for their regeneration into entire plants. Protoplasts, by virtue of the conditions under which they are prepared, are unavoidably exposed to shifts in their osmotic environment, pH and other factors which may be deemed detrimental. Consequently, the effect that protoplast isolation has on the plant protein synthesizing system has been assessed. In addition, cation and polyamine treatments which have been shown to generally improve protein synthesis *in vitro* (77,136) were evaluated in order to determine optimal conditions for barley protoplast protein synthesis. The response of protoplast protein synthesis and degradation to metabolic inhibitors and light was also examined.

Osmotic Stress and pH

The protein synthetic capabilites of '400', '500' and '700' protoplasts were compared over a 4 h incubation period. The '700' protoplasts incorporated radiolabelled methionine into protein at an almost linear rate for 2 h (Fig. 3). In contrast, the rate and extent of radiolabelled amino acid incorporation by '400' and '500' protoplasts was much lower. The rate of protein synthesis varied between different preparations but was consistently found to be linear for 60 min in the case of '700' protoplasts. Therefore, a 60 min incubation period was chosen for all further protein synthesis assays.

As the synthetic activity varied considerably between protoplast preparations (replicates), protein synthesis data is expressed as a percent of the control. The data, as a percent of control, were analyzed by analysis of variance and are presented. However, for the data in Table V where control values are included, the raw data was transformed using logarithms before being subjected to analysis of variance (32).

There was a consistent decrease in the protein synthetic capabilities of protoplasts with a decrease in the osmotic concentration of the buffer in which they were suspended (Table V). These results contradict the data in some reports (decreased protein synthesis with increasing osmotic concentration of the suspending buffer) (187,204) yet are in agreement with other studies (58,266). These experiments tested the effect of osmotic concentration on protein synthetic capacities of plant protoplasts or cells. Anomalous amino acid uptake could be considered as a possible explanation for the above results. However, previous research (187,266) has demonstrated that reduced rates of amino acid uptake could not explain the lower rates of protein synthesis by protoplasts suspended in buffer of different osmotic concentrations. Amino acid uptake by the different protoplast 'types' was not evaluated in the present study.

The maximum rate of radiolabelled amino acid incorporation in this study was 750,000 cpm $h^{-1}10^{-4}$ protoplasts (925,000 cpm $h^{-1}\mu g$ chl^{-1}). This is approximately equivalent to 6 fmoles of methionine incorporated per minute by 10^4 protoplasts. The rate of protein

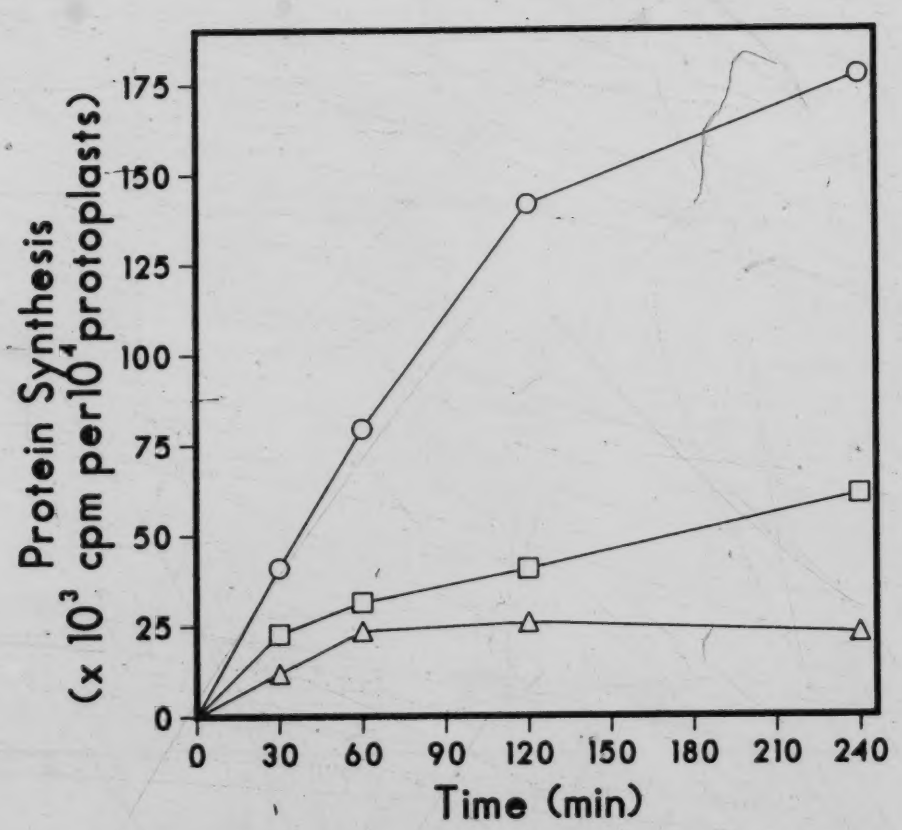


Figure 3. Synthesis of proteins by protoplasts incubated in a range of sorbitol concentrations. All protoplasts were incubated under light at 25°C with 6.5 μCi of [^{35}S]-methionine per 10^4 protoplasts. Values are averages of 2 replicates. '400' (△), '500' (□), '700' (○) protoplasts.

Table V. Protein synthesis by protoplasts isolated and incubated in a range of sorbitol concentrations. All protoplast treatments were done under light at 25°C with 6.5 μCi of [^{35}S]-methionine per 10^4 protoplasts. Inhibitor treatments were 100 μg/ml chloramphenicol or 25 μg/ml cycloheximide. Logarithims of raw data were analyzed by analysis of variance. Values are geometric means of 5 replicates expressed as cpms of [^{35}S]-methionine incorporated into TCA-preciptated protein after 60 min by 10^4 protoplasts.

	Concentration of Sorbitol (mM)				
Treatment	400	500	600	700	Means
Control	8043	28375	41816	62697	27812a
Cycloheximide	6003	19353	26500	32740	17818b
Choramphenicol	2210	9316	13844	22813	8980c
Means	4744 a	17231 b	24848 c	36041 d	

Treatment x concentration interaction is not significant at the 5% level.
All means, within treatments or concentrations, followed by the same letter are not significantly different at the 5% level as determined by LSD.

synthesis by barley protoplasts in this study was four times greater than that reported for spinach (165) and 50 times less than an earlier result for tobacco (205).

CAP and CHI are relatively specific inhibitors of chloroplastic and cytoplasmic protein synthesis, respectively (66). Possible secondary effects exerted by these inhibitors on other metabolic processes can be minimized by using a short assay period. In this study, an evaluation was carried out to determine the specificity of CAP and CHI on barley protoplast protein synthesis. This specificity was assessed both by the extent of inhibition observed for either inhibitor alone or together in addition to analysis of the nature of the polypeptide products synthesized. When CAP and CHI were used simultaneously no protein synthesis occurred in barley protoplasts. Furthermore, the protein synthesis rates in the presence of the inhibitors used individually summed to approximately the total untreated protein synthetic rate (Table V, rows 2+3=1). Another important observation regarding inhibitor specificity can be drawn from the SDS-PAGE polypeptide profiles generated from samples treated with

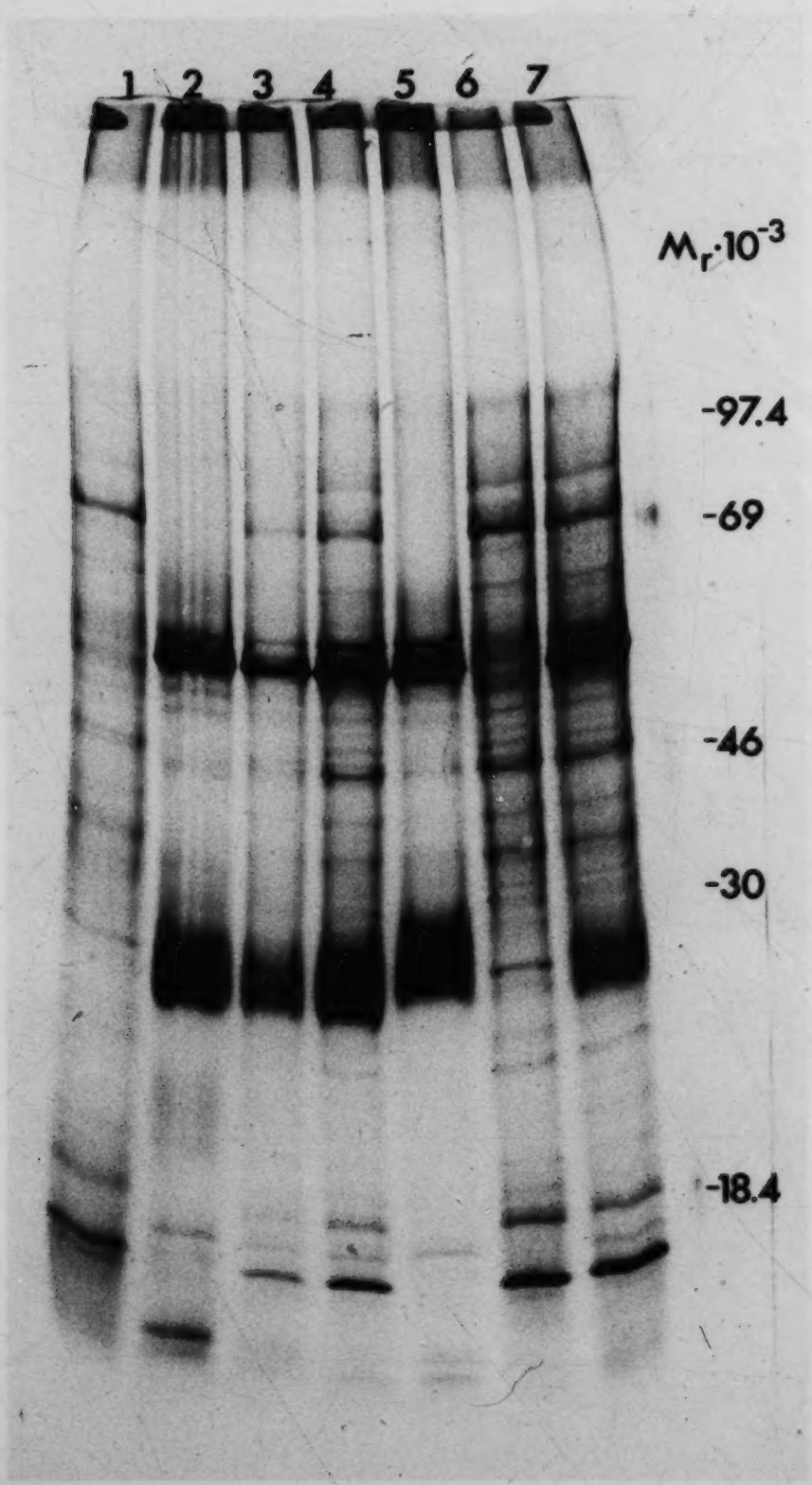
inhibitors (Fig. 4). In the presence of CAP (lanes 1 and 6), the LSU of Rubisco (55 kD) and the HBP (broad band at 30 kD) are not synthesized. In turn, CHI treatment (lanes 2 and 5) completely suppresses the synthesis of the nuclear-encoded pSSU of Rubisco (14 kD). In addition, polypeptides migrating at 60, 57, 16, 12 and 11 kD are specifically inhibited by CAP (lanes 1 and 6). Polypeptides migrating at 82, 72, 66, 46, 42, 38, 34, 23 and 19 kD are not synthesized in the presence of CHI (lanes 2 and 5). Thus, the inhibitors CAP and CHI exhibit similar consequences in barley protoplasts as found with other plant protoplast systems (94,204,205).

High rates of chloroplastic protein synthesis were measured with '700' protoplasts incubated with 25 μg/ml CHI. Methionine incorporation by the chloroplasts in these barley protoplasts was 300 fmoles μg chl^{-1}h^{-1}. This rate is equivalent to the highest reported rate of amino acid incorporation by isolated chloroplasts (175).

Previous reports on protoplast protein synthesis demonstrated that cytoplasmic protein synthesis was inhibited to a greater extent than chloroplastic protein synthesis (59,58,94). The reason for the preferential inhibition of cytoplasmic protein synthesis in these studies is not known. High osmotic concentration has been implicated for reduced rates of total protein synthesis in protoplasts (59,187). Therefore, for this study it was assumed that differential rates of protein synthesis by the chloroplasts and the cytoplasm would be evident when the protoplasts were incubated at different osmotic concentrations. The lack of a significant interaction in Table V suggested that the relative contribution by chloroplasts to total protein synthesis did not vary with protoplast 'type'. This observation was also true if the data were presented and analyzed on a per μg chl basis.

If cytoplasmic protein synthesis is suppressed in protoplasts then the relative contribution of the chloroplasts and the cytoplasm to total protein synthesis may differ between protoplasts and cells or leaves. Table V demonstrates that chloroplasts of barley protoplasts contribute (assuming that contibutions by mitochondria are negligible, 74) up to 68% of the total protein synthesized during a 60 min incubation period. Similar values derived

Figure 4. Fluorograph of proteins synthesized by '500' (lanes 1,2,3) and '700' (lanes 4,5,6,7) protoplasts in the presence of protein synthesis inhibitors. Protoplasts were incubated with 6.5 μCi [^{35}S]-methionine per 10^4 protoplasts for 60 min and separated by SDS-PAGE. Lanes 1 and 6 are proteins synthesized in the presence of 100 μg/ml CAP and lanes 2 and 5 are proteins synthesized in the presence of 25 μg CHI. Lanes 3 and 4 are proteins synthesized by untreated protoplasts. Lane 7 is protein synthesized by '700' protoplasts in the dark. Each lane was loaded with equal cpm of [^{35}S]-methionine-labelled products.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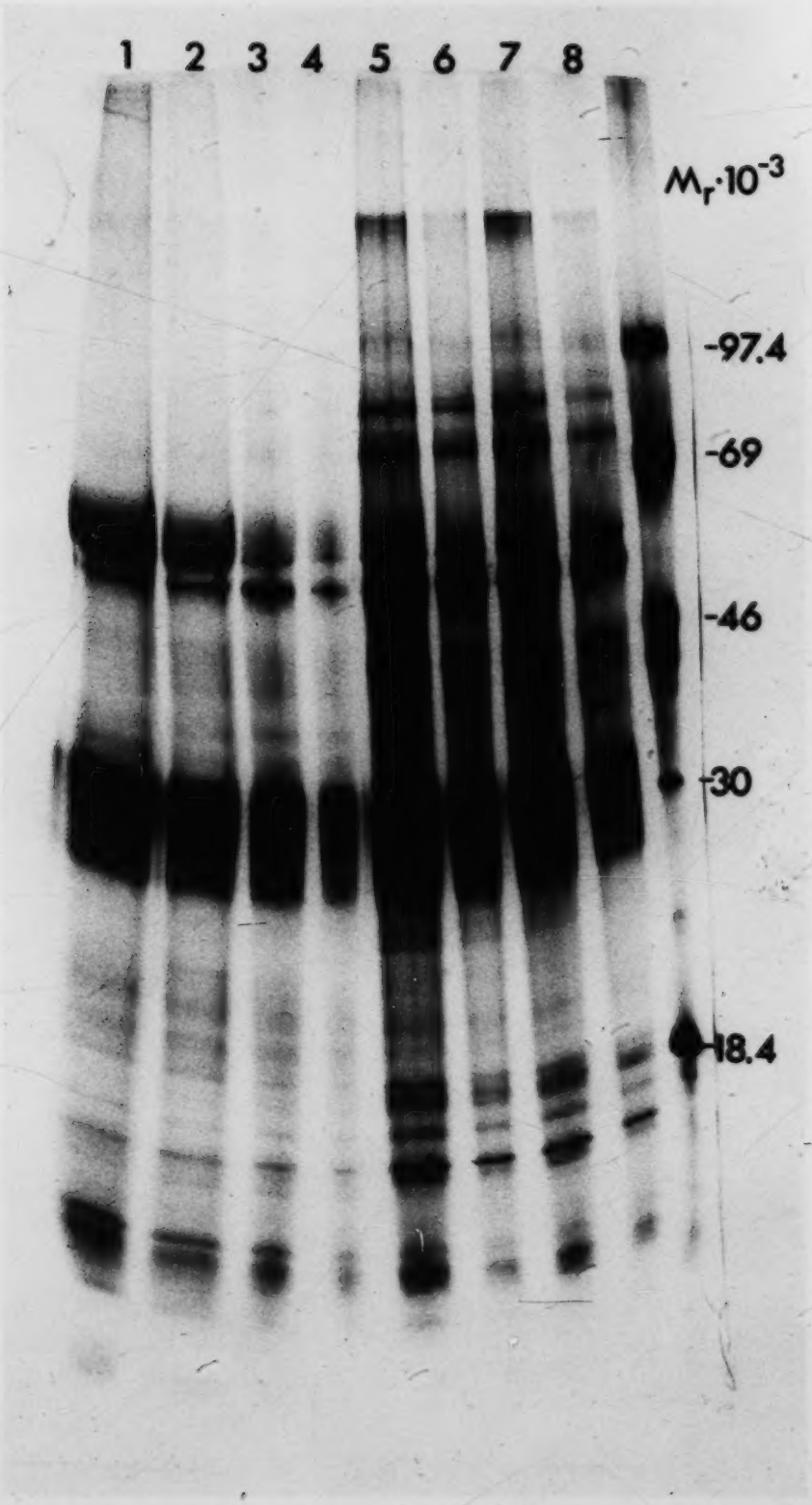
5
6
7
$M_r \cdot 10^{-3}$
-97.4
-69
-46
-30
-18.4

from *in vivo* measurements for pea shoots (24) and algal suspensions (195) are 36 and 40%, respectively. These somewhat lower values are in agreement with those obtained using tobacco protoplasts but only when a 30 min incubation period is considered (94). Interestingly, cytoplasmic protein synthesis by tobacco protoplasts declines dramatically after 30 min so that by 90 min the contribution of this fraction to total protein synthesized is only 30%. By difference, it can be seen that chloroplasts now account for 70% of total protein synthesis in closer agreement to the data for barley protoplasts. These differences reported for the various organisms are not due to variable uptake of inhibitors by the different systems (94,195). Therefore, the relative contribution of the cytoplasm to total tobacco protoplast protein synthesis appears to decrease with length of incubation. Further experiments are required in order to determine the cause of the decrease in cytoplasmic protoplast protein synthesis and to determine if this relationship applies to barley protoplasts as well.

Fleck and her co-workers (58) found qualitative differences between the polypeptide products synthesized by protoplasts, cells and leaves. Again the effect that different osmotic concentrations might have on protoplast protein synthesis was used to try to explain any differences observed in the proteins synthesized by protoplasts and those synthesized by intact leaf tissue. Figure 4 shows no discernible differences among the complement of proteins synthesized by '500' and '700' protoplasts despite the fact that the '500' protoplasts analyzed on this gel were half as efficient at synthesizing protein as the '700' protoplasts (Table V). The gels were loaded with equal amounts of radioactivity, and yet small quantitative differences between '500' and '700' protoplasts can be seen for all protein bands. These minor distinctions are not a function of the protoplast preparation or the gel since the same dissimilarities were reproducible on other gels (including Fig. 5). Figure 5 which displays SDS-PAGE polypeptide patterns for all four protoplast 'types,' also exhibits subtle decreases in the quantities of all '400' (lanes 1 and 2) and '500' (lanes 3 and 4) protoplast polypeptide products. It is important to note that all protoplast 'types' synthesize the same complement of proteins.

Figure 5. Fluorograph of proteins synthesized by '400' (lanes 1,2), '500' (lanes 3,4), '600' (lanes 5,6) and '700' (lanes 7,8) protoplasts. Protoplasts were incubated for 60 min at 25°C with 6.5 μCi of [^{35}S]-methionine per 10^4 protoplasts and labelled proteins were separated by SDS-PAGE. Lanes 1,3,5,7 were loaded with 50,000 cpm and lanes 2,4,6,8 were loaded with 25,000 cpm of [^{35}S]-methionine-labelled products.

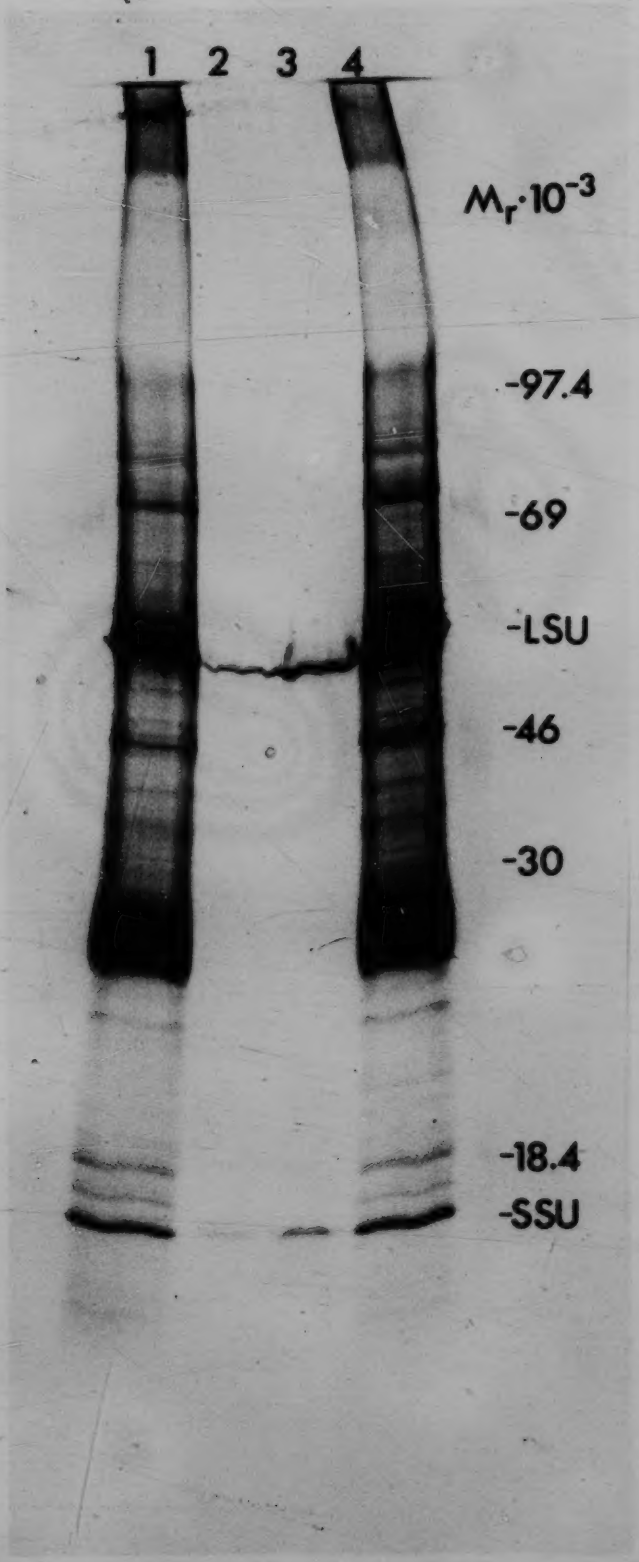

1 2 3 4 5 6 7 8
$M_r \cdot 10^{-3}$
-97.4
-69
-46
-30
-18.4

An antibody directed against the Rubisco holoenzyme was used to enable a more specific qualitative and quantitative analysis of the cytoplasmic and chloroplastic contributions towards protein synthesis. Figure 6 (lanes 2,3) illustrates that, as a fraction of total protein synthesized, there is less SSU and LSU synthesized by the '500' protoplasts. However, within each barley protoplast 'type' the ratio of LSU to SSU synthesized is similar. In conclusion, osmotic concentration quantitatively affects protein synthesis by barley protoplasts. The data presented in Fig. 4,5,6 and Table V also indicate that osmotic concentration affects cytoplasmic and chloroplastic protein synthesis equally. Therefore, the differential inhibition of cytoplasmic protein synthesis in tobacco protoplasts (58) is not likely a function of osmotic concentration.

Figure 6 also confirms that the 55 kD and 14 kD protein bands are indeed the LSU and SSU of Rubisco, respectively. Furthermore, as the antibody did not precipitate a polypeptide of a Mr of 20 kD it is assumed that the pSSU was processed. Since processing of the pSSU has been shown to occur in the chloroplast stroma (223) it is further assumed that the pSSU was transported into the chloroplasts of the barley protoplasts.

Although protoplasts subjected to different osmotic conditions display similar patterns of synthesized proteins (Fig. 4,5,6), there are a number of reports that proteins synthesized by protoplasts are lacking various proteins synthesized by cells or leaves (58,59,68,148,203,204). It is not possible to compare rates of protein synthesis in protoplasts to those of cells or leaves because of substantial variations which exist between rates and methods of radiolabelled amino acid uptake. In this study, barley cells and protoplasts were prepared in parallel using 700 mM sorbitol as the osmoticum. Identical methodology was employed except the digesting buffer used to release cells lacked cellulase. The cells, with their cell walls intact, appear distorted and spongey (Fig. 7) and resemble previously published light-micrographs of tobacco mesophyll cells (105,176,234). Cells were incubated with [^{35}S]-methionine for 2 h as previously described for protoplasts. Protein synthesis by the cells was poor (9,000 cpm/10^4 cells) which may be a result of inadequate methionine uptake

Figure 6. Immunoprecipitation of LSU and SSU of Rubisco from proteins synthesized by '500' and '700' protoplasts. Lanes 1 and 4 contain proteins synthesized by '500' and '700' protoplasts, respectively. Immunoprecipitates of '500' and '700' protoplast protein products are in lanes 2 and 3, respectively. Equivalent cpms of [^{35}S]-methionine-labelled products were used for each immunopreciptation and the same number of cpms was loaded in lanes 1 and 4.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4
$M_r \cdot 10^{-3}$
-97.4
-69
-LSU
-46
-30
-18.4
-SSU

through the cell wall (178). Polypeptide products were also prepared from excised leaves of 6 d barley. The severed ends of three leaves were placed in 20 μl of 700 mM sorbitol buffer containing 130 μCi of [^{35}S]-methionine. Uptake and incorporation of methionine under light (PAR 350 μE $m^{-2}s^{-1}$) proceeded for 2 h before leaves were ground in a tissue grinder and the 500*g* supernatant diluted with SDS-PAGE solubilizing buffer.

The polypeptide products from leaves, cells and protoplasts are compared in Figure 8. The proteins synthesized by each system are, indeed, quantitatively and qualitatively different from each other and, apparently, different from the patterns reported by Fleck *et al.* (58,59). Barley cells synthesized very little LSU and HBP (Fig. 8, lane 1). These two proteins were synthesized very well by tobacco cells held in isotonic or hypertonic buffers (58). The SSU is synthesized in small quantities by barley cells and protoplasts (Fig. 8, lanes 2 and 3). The SSU has been variously reported to be either synthesized (94) or not synthesized by tobacco protoplasts (59).

A much broader array of proteins were labelled by severed barley leaves (Fig. 8, lane 4). The LSU and especially the SSU are synthesized in much greater quantity by the severed leaves than by either the protoplasts or the cells (Fig 8). A number of other polypeptides are also synthesized in much greater quantities by severed leaves than by the protoplasts. By way of comparison to other gels (eg. Fig.4), polypeptides at Mr 102, 72, 46, 42, 38 and 23 kD which are synthesized by severed leaves only, appear to be of cytoplasmic origin. However, cytoplasmically synthesized polypeptides at Mr 19 and 66 kD are synthesized by both protoplasts and leaves (Fig. 8, lanes 3,4). Furthermore, the synthesis of the HBP by leaves is markedly less than that found for protoplasts. A number of studies using severed leaves suggest the HBP, expressed as a percent of total protein or thylakoid protein, is synthesized in reduced amounts (173,24) as compared to estimates drawn from analogous experiments using suspensions of aquatic plant species (195,192,259). Also, the number of different proteins which are synthesized in large quantities by aquatic plants in suspension is less than that synthesized by intact leaves. These two points suggests that protoplast protein

Figure 7. Light-micrographs of cells (a) and protoplasts (b) isolated from 6 d barley leaves. The cells and the protoplasts were both isolated using 700 mM sorbitol buffer, however the digestion buffer for the cells did not contain cellulase. The bars represent 25 μm.

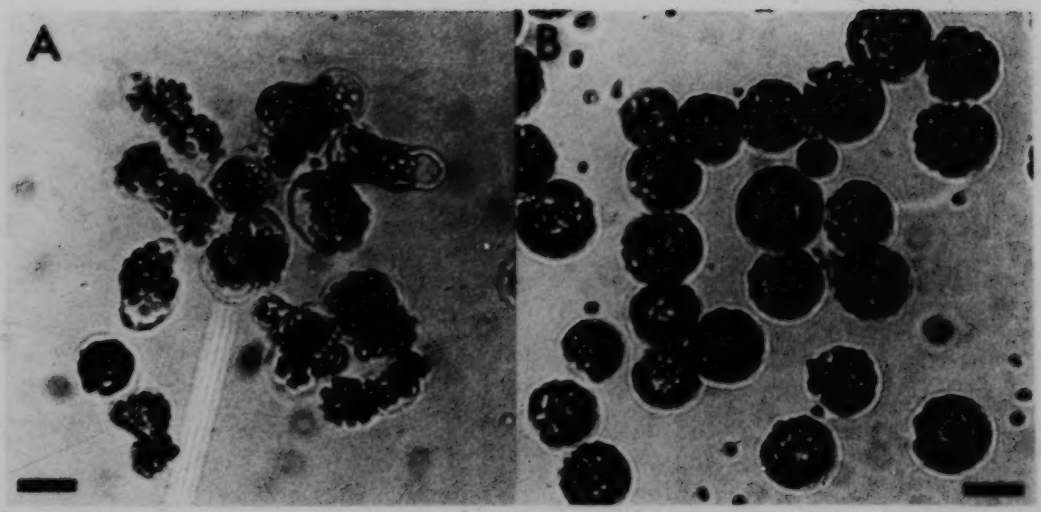
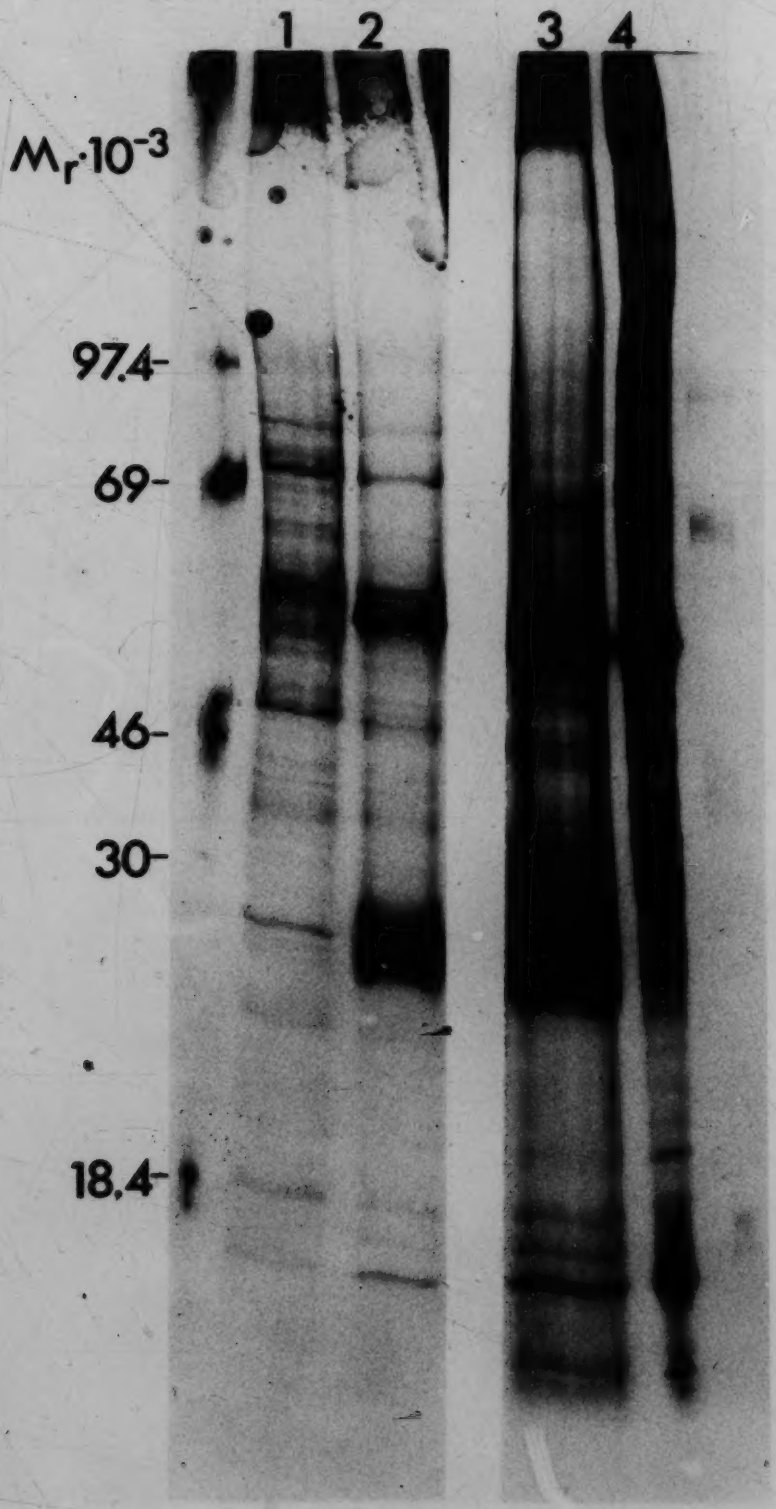

A
B

Figure 8. Fluorograph of proteins synthesized by cells, protoplasts and leaves of barley. Lanes 1 and 2 were loaded with proteins synthesized by cells and protoplasts, respectively. Lanes 3 and 4 were loaded with proteins synthesized by protoplasts and leaves, respectively. Each pair, lanes 1,2 and 3,4 , was loaded with equal cpms and exposed to X-ray film for an equivalent length of time. See 'Results and Discussion' for methods of labelling.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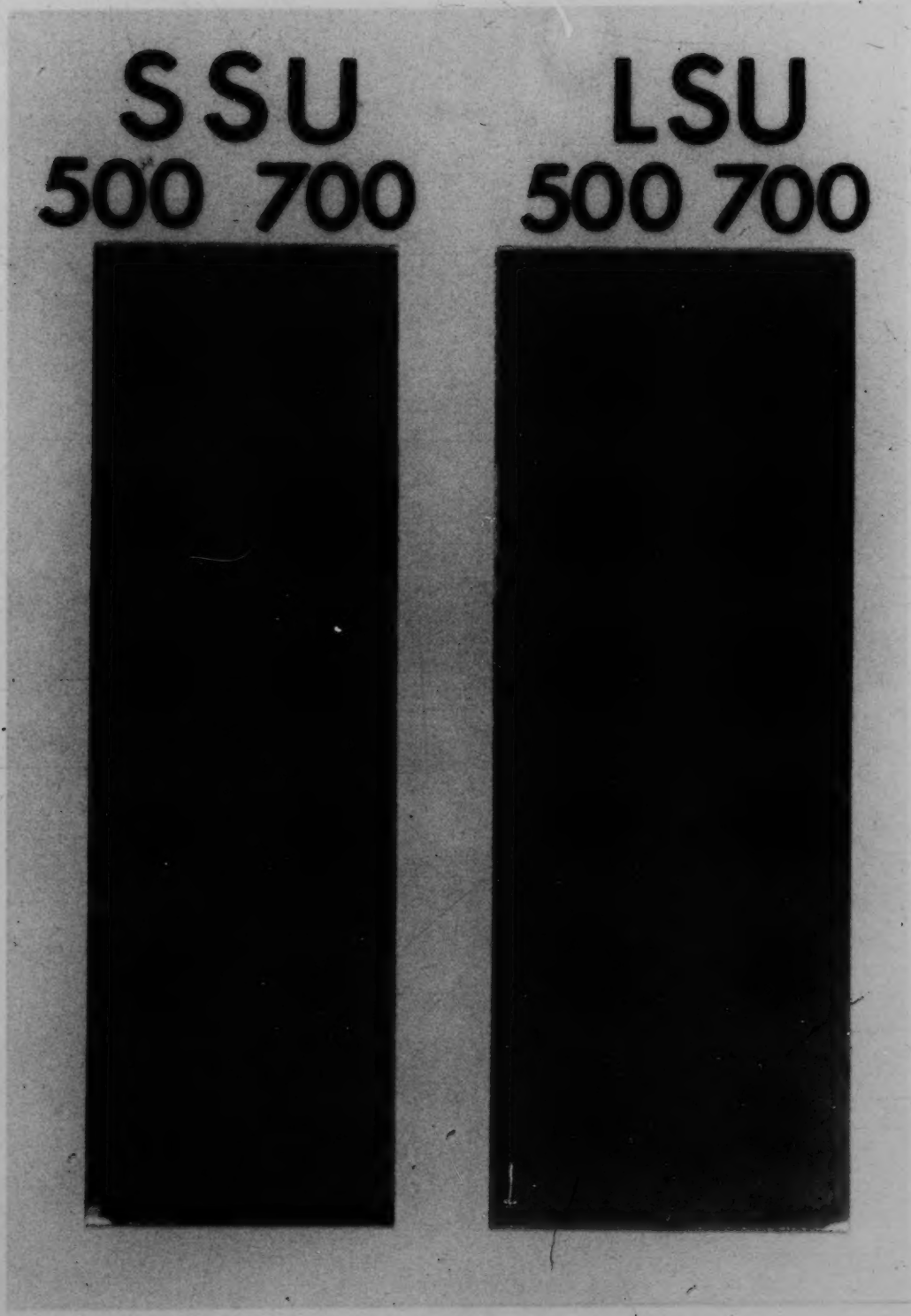
30-
18.4-

synthesis patterns are more related to those shown for aquatic species than those of severed leaves. However, no definite conclusions can be drawn on whether the pattern of polypeptides synthesized by protoplasts or severed leaves is more closely related to the *in vivo* situation. Cytoplasmic protein synthesis does not appear to be preferentially inhibited in protoplasts since some cytoplasmic polypeptides are synthesized by the barley protoplasts (Fig. 8). Aquatic species like *Euglena, Spirodela, Chlamydomonas* or *Olisthodiscus* are the only plant systems in which the labelling of plant proteins can be considered to be truely *in vivo* and not subject to unavoidable conditions of stress. It would be of interest to compare protoplasts obtained from aquatic species subjected to different osmotic concentrations to the intact plant, in order to determine if any changes in metabolism occur that can be attributed solely to osmotic environment.

In the previous discussion only the effects of 'protoplast stress' on translation have been addressed. Indeed, reduced protein synthesis could be due to reduced mRNA levels. After 2 h of incubation total RNase activity is not affected by osmotic concentration (Table I). However, '500' protoplasts which have been incubated for more than 2 h may have higher levels of total RNase activity (Table III) and therefore decreased rates of protein synthesis in these protoplasts may be attributed to decreased quantities of mRNA.

Plasmids containing LSU and pSSU mRNA were hybridized to total RNA extracted from 6 h old '500' and '700' protoplasts. There was no significant difference in the quantities of Rubisco transcripts between the '500' and '700' protoplasts (Fig. 9). Therefore, the effect of osmotic concentration on barley protoplast protein synthesis does not appear to be related to the availability of mRNA transcripts. As a percentage of total RNA the LSU mRNA is more abundant than the pSSU mRNA in extracts from both protoplast 'types'. Vernet *et al.* (245) quantitated the pSSU mRNA from leaves and freshly prepared protoplasts of tobacco. They found negligible amounts of pSSU mRNA in the poly(A) RNA fraction of protoplasts. This result is surprising since the tobacco protoplasts were 'freshly isolated', viable and capable of regeneration whereas the barley protoplasts used in this study were 6 h old and not

Figure 9. Autoradiographs of dot-blot hybridizations of plasmids containing the LSU and SSU of Rubisco against total RNA extracts from '500' and '700' protoplasts. Nylon membranes were spotted with 25, 10, 5, 2, 1 and 0.5 μg of total RNA.


SSU
500 700
LSU
500 700

viable for extended periods (Tables I and II). It would be interesting to know whether Vernet and co-workers would have obtained the same result if a total RNA extract had been probed as was done for the barley protoplasts in this study. The cDNA probes used in Figure 9 would hybridize to Rubisco mRNA of all sizes including mRNA which completely lacks a poly(A) tail. The purpose of mRNA poly(A) is still not known (19) but it has been demonstrated that the poly(A) sequence does degrade over time (19). Consequently, pSSU and other polyadenylated transcripts may be present in lower quantities in a protoplast poly(A) RNA fraction if rates of poly(A) degradation are increased or rates of transcription are reduced. The extent to which mRNA is polyadenylated does not appear to affect the translatability of the mRNA (19). Therefore, the mRNA in a total RNA extract as opposed to a poly(A) RNA preparation may give a better indication of the mRNA available for translation.

The enzymes used to isolate protoplasts have optimal activities at pH 5.0 (203). The normal pH of the intercellular sap of plants lies between pH 5 and 6 (221). Therefore, it is not surprising that most protoplasts survive best at pH 5.5 to 6.0 (203,225). In contrast, the pH of the cytoplasm is pH 7 to 8 (84) and most research with intact chloroplasts is done at pH 7.8 (56,189).

Protein synthesis by barley protoplasts at pH 5.5 and 7.8 was compared employing [^{35}S]-methionine and [^{3}H]-leucine. Protein synthesis using [^{35}S]-methionine at pH 7.8 was only 32±5% (SE) of that achieved at pH 5.5. No incorporation of leucine occurred at pH 7.8. Leucine was incorporated into protein by protoplasts at pH 5.5 at the rate of 200 fmoles μg chl^{-1}h^{-1}. This rate is similar to that calculated for methionine incorporation into protein. A decrease in uptake of the amino acids may have greatly reduced protein synthesis at pH 7.8. Indeed the uptake of leucine by *Vinca rosea* protoplasts at pH 8.0 was 50% of that at pH 4 (232). A greater effect of pH on the uptake of AIB, serine and glutamine was reported for soybean protoplasts (246). In this study amino acid uptake by protoplasts was eight times most readily at pH 5 than at pH 8 (246). Many compounds will permeate the plasmalemma greatest at a pH near their pI (33). The pI of leucine and methionine are 6.02

and 6.74, respectively.

Treatments with Cations and Metabolic Inhibitors

Various metabolic reactions including protein synthesis have specific requirements for a number of cations. Potassium is required for ribosome assembly (77,153) and also plays a role as a counter-ion in ATP-mediated transport (132,191). Calcium is inhibitory to *in vitro* protein synthesis (77) but appears to stabilize the plasmalemma (2,203,243) and be involved in active transport of a variety of compounds across the plasmalemma (99,132,221). Magnesium improves translation fidelity (1) and is an important counter-ion in the chloroplast (84). In addition, all the above cations are required as cofactors for numerous enzymatic reactions (84). Acetate has been recommended as the counter-ion of choice for *in vitro* protein synthesis (77) since chloride has proved to be inhibitory in studies of *in vitro* protein synthesis (77,253).

Reports on the effect of cations on protoplast protein synthesis are limited. Therefore various cations were tested for their ability to influence protoplast protein synthesis. All the acetate salts were very inhibitory to protoplast protein synthesis (Table VI). Since acetate is not known to interfere with the protein synthesis apparatus or with protein synthesis reactions per sé (77), the effect of acetate is probably due to indiscriminate acetylation of lysyl-ϵ-amino groups of membrane proteins (34). This acetylation can reduce sugar uptake at concentrations of less than 2 mM acetate (99).

When compared to the untreated control, all concentrations of $MgCl_2$, $CaCl_2$ and KCl that were tested did not significantly affect protein synthesis (Table VI). Other reports have shown that 100 mM KCl or $CaCl_2$ can inhibit amino acid uptake by protoplasts (204). As the chloride salts are not inhibitory they could be included in protoplast buffers at a low concentration as a precautionary measure against possible ion loss during protoplast isolation.

The herbicides atrazine, diuron, simazine and chlorsulfuron permeate the plasmalemma rapidly (33,38). These herbicides did not, however, affect protoplast protein

Table VI. Effects of various cations and anions on protoplast protein synthesis. Protein synthesis was carried out with 6.5 μCi of [^{35}S]-methionine per 10^4 protoplasts for 60 min under light at 25°C. Values are means of 3 replicates expressed as a percentage of the untreated control. The average amount of [^{35}S]-methionine in TCA-preciptable protein of the untreated control was 115,000 cpm/10^4 protoplasts.

Salt	Concentration (mM)	Percent of Control
$MgCl_2$	1.0	126a
	10.0	72cd
KCl	0.1	122a
	1.0	109ab
	5.0	98abc
	10.0	111ab
$CaCl_2$	1.0	79bcd
	10.0	82bcd
K-acetate	1.0	102abc
	10.0	2e
Ca-acetate	1.0	74cd
	10.0	0e
Mg-acetate	1.0	63d
	10.0	0e

All means followed by the same letter are not significantly different at the 5% level as determined by LSD.

synthesis (Table VII). The slight reduction in protein synthesis observed for simazine can be attributed entirely to the methanol in which the herbicide was dissolved. Diuron, simazine and atrazine are photosynthetic electron transport inhibitors which bind directly to or near the HBP (103,242). Their action should lead to the eventual depletion of ATP and reducing equivalents generated by activities of chloroplasts. However, as these compounds have no apparent effect on protoplast protein synthesis (Table VII), there must be a sufficient supply of ATP and reducing equivalents in the protoplasts to carry out protein synthesis. Chlorsulfuron inhibits the biosynthesis of valine and isoleucine in plants and bacteria (190). This chemical does not inhibit protein synthesis so it is expected that the pool of these branched-chain amino acids must not be limiting during the 60 min incubation period.

Table VII. Effect of inhibitors, betaine and RNase on protoplast protein synthesis. Protein synthesis was carried out with 6.5 μCi of [^{35}S]-methionine per 10^4 protoplasts for 60 min under light at 25°C. Values are means of 3 replicates expressed as a percentage of the untreated control. The average amount of [^{35}S]-methionine in TCA-precipitable protein of the control was 253,245 cpm/10^4 protoplasts.

Treatment	Concentration	Percent of Control
Betaine	500 μM	112a
Chlorsulfuron	1 μM	103ab
Atrazine	20 μM	100ab
Diuron	20 μM	92abc
Methanol	1% (v/v)	87abc
Simazine •	20 μM	84bc
RNase (bovine pancreatic)	20 μg/ml	71cd
DCCD •	100 μM	49d

• treatment contains 1% (v/v) methanol.
All means followed by the same letter are not significantly different at the 5% level as determined by LSD.

DCCD (N,N'-dicyclohexyl carbodiimide) inhibits oxidative phosphorylation and photophosphorylation (103). DCCD also inhibits the active transport of ions (145), sugars (133) and leucine (232) across the plasmalemma by modifying membrane proteins involved in transport. Protein synthesis was markedly inhibited by DCCD in this study (Table VII). This inhibition may be due to a shortage of ATP since ATP production in both organelles would likely be hindered. Alternatively, DCCD may interfere with the uptake of radiolabelled methionine by the protoplasts.

Ribonuclease (bovine pancreatic) inhibited protoplast protein synthesis by 29% (Table VII). The survival of oat protoplasts was also reduced by pancreatic RNase (67). RNase should not be able to enter intact protoplasts or organelles (51). In this study the RNase may be acting on ruptured protoplasts or the RNase preparation (from Sigma) may contain contaminating proteins or salts which may affect protoplast protein synthesis or membrane intactness.

Betaine accumulates to levels as high as 250 μmoles/g DW in the chloroplasts and cytoplasm of plants subjected to salinization or water stress (87,199). Therefore, betaine may play a role in alleviating osmotic stress. In the present study, 500 μM betaine did not affect protoplast protein synthesis (Table VII). However, even though betaine appears to be transported between the chloroplasts and the cytosol (125,199), it is not known whether betaine was taken up by the protoplasts in this study.

Polyamines

Polyamines are often included in *in vitro* protein synthesis assays to improve both the rate and fidelity of translation (1,77,172,233). The actual mechanism by which polyamines improve *in vitro* protein synthesis is believed to involve the stabilization of nucleic acids through the neutralization of the negative charges on the phosphate groups of nucleic acids (233). Polyamines have also been used to retard leaf senescence and chlorophyll loss (30,113,182) in addition to improving the survival of plant protoplasts (67,2).

The literature dealing with polyamine improvement of protoplasts contains many discrepancies. Galston and his co-workers were able to improve protoplast protein synthesis by pretreating leaves with the basic amino acids lysine and arginine (109). However, when the protoplasts were directly treated with 1 or 10 mM arginine they exhibited decreased rates of protein synthesis (2). Direct effects of exogenously added putrescine, cadaverine, spermidine or spermine on protoplast protein synthesis have not been reported.

Various amines including the indoleamine, gramine, were examined to assess their effect upon protoplast protein synthesis. None of the amines at 0.001 or 0.01 mM significantly improved barley protoplast protein synthesis (Fig. 10). The uptake and incorporation of [^{35}S]-methionine by the protoplasts was inhibited in the presence of 1 mM concentrations of all amines execpt the diamine cadaverine (Fig. 10). In general, the more positively charged tetra-amine, spermine, was the most inhibitory. However, spermine, the triamine, spermidine, and the indoleamine, gramine, were all capable of inhibiting protoplast

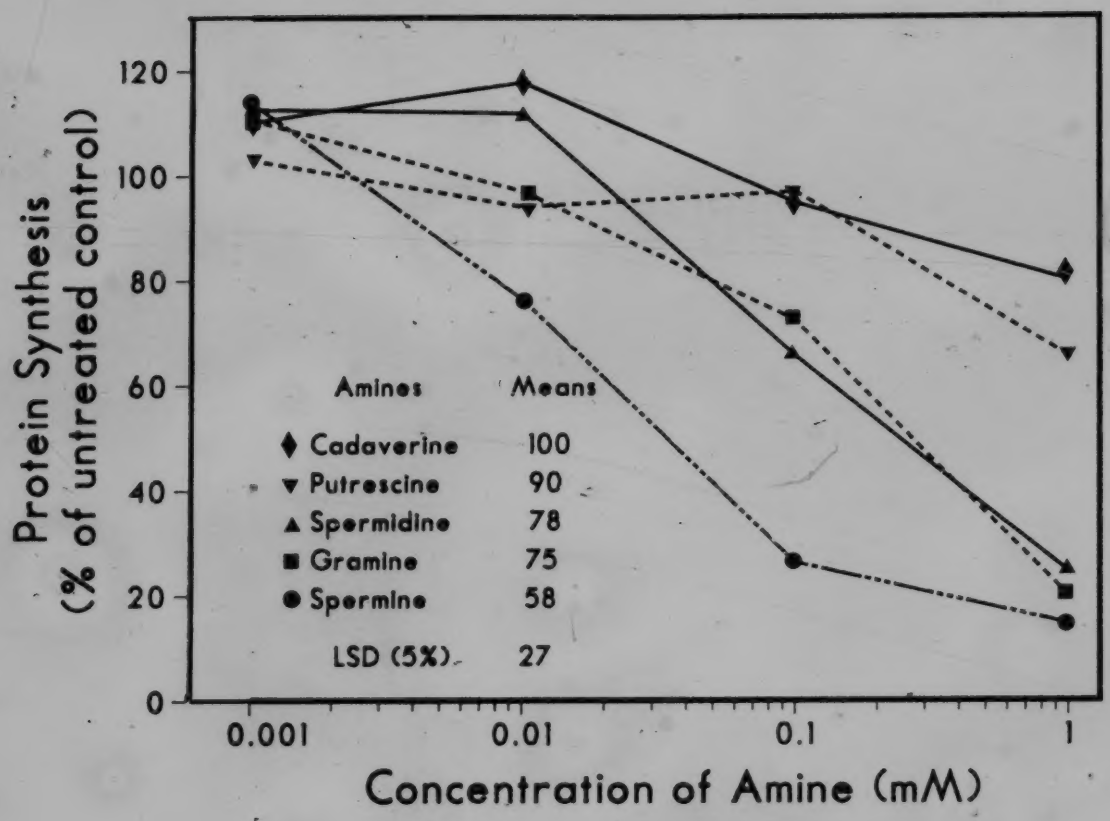


Figure 10. Inhibition of protoplast protein synthesis by amines. Protoplasts from 6 d barley laminae were isolated and incubated in 700 mM sorbitol buffer. Protein synthesis was carried out with 6.5 μCi of ^{35}S-methionine per 10^4 protoplasts for 60 min under light at 25°C. Values are means from 3 replicates, expressed as percentages of the untreated control. The average amount of ^{35}S-methionine in TCA-precipitates of the control was 122,000 cpm per 10^4 protoplasts. The means for the amine concentrations were 110, 99, 71 and 41% respectively for 0.001, 0.01, 0.1 and 1.0 mM. The LSD value for amine concentration at the 5% level was 24. The LSD value for amine x concentration interaction at the 5% level was 27.

protein synthesis by approximately 80% at a concentration of 1 mM. Gramine, at concentrations of 1 and 10 mM, is reported to be toxic towards calli of some barley varieties (86). The inhibition of protein synthesis appears to be associated with the degree of amination of the compounds. This suggests that the amine groups are responsible for the observed inhibition.

Figure 11 illustrates the effect that 1 mM spermine has on protoplast protein synthesis over a 120 h time period. Protein synthesis in the presence of 1 mM spermine appears to be completely inhibited after 45 min of incubation. The polyacrylamide gel in Figure 12 shows the array of polypeptides synthesized in the presence of spermine. All 3 major proteins are synthesized (lanes 3,4) with the LSU being represented more prominently among the products synthesized than either the HBP or SSU. When this gel was overexposed no specific protein bands were missing from the spermine treated protoplasts.

Three possible explanations can be entertained to explain the results obtained in Figure 10. The first explanation might be that the effect observed is not a direct consequence of the polyamine tested but rather an indirect result of the spermine formulation. Spermine can be obtained as a free base or as a chloride salt. The di-, tri- and tetra-amines used in the preceeding experiment were formulated as chloride salts. Therefore, in addition to spermine possessing more amine groups, a solution made from spermine tetrahydrochloride would contain more free Cl^- than the other amine solutions. Chloride is known to inhibit *in vitro* protein synthesis (253) and its influence on protoplast protein synthesis was tested. Spermine formulated as a free base was compared to spermine tetrahydrochloride (Table VIII). Both formulations were equally inhibitory to protein synthesis. In addition, inhibition was observed regardless of how the stock solution of spermine was buffered (acetic acid versus HCl) or if it was buffered at all (Table VIII). Therefore, as was illustrated in Table VI with the use of various salts, chloride does not appear to inhibit protein synthesis in barley protoplasts at the concentrations tested.

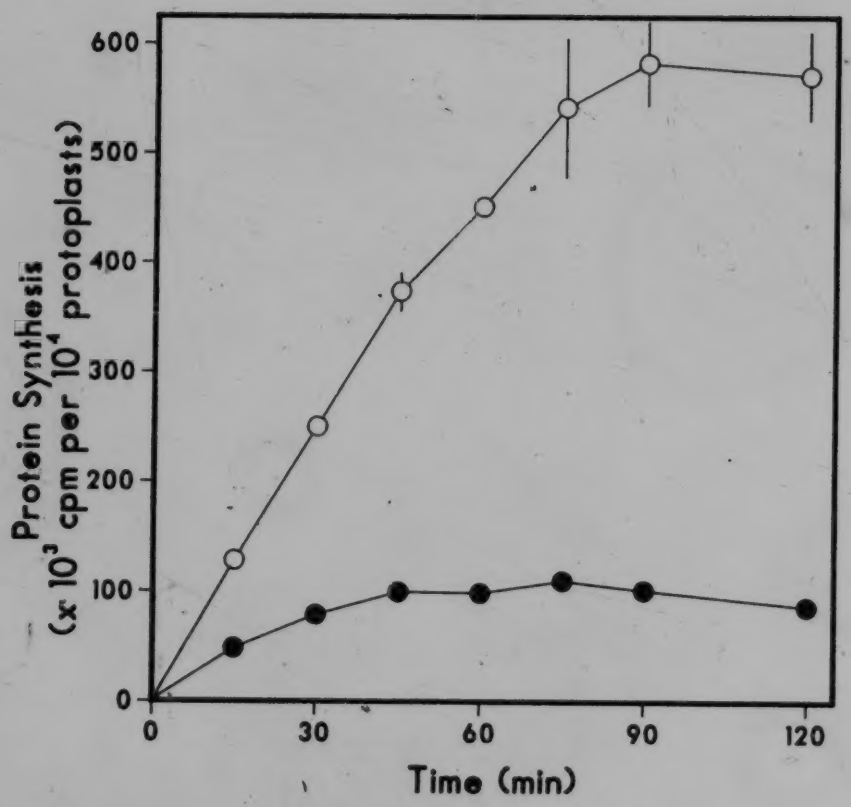


Figure 11. The effect of spermine on protein synthesis in protoplasts. Protoplasts ('700') were incubated with 6.5 μCi [^{35}S]-methionine per 10^4 protoplasts under light at 25°C. Values are means of TCA-precipitable protein expressed as cpms from 3 repeats drawn from the same suspension of protoplasts. Untreated (○), 1 mM spermine (●)

Figure 12. Fluorograph of proteins synthesized in protoplasts in the presence of spermine. Protoplasts were incubated with 6.5 μCi of [^{35}S]-methionine per 10^4 protoplasts for 60 min at 25°C under light. Labelled proteins were separated by SDS-PAGE. Lane 1 is protein from untreated control '700' protoplasts. Lane 2 is protein from protoplasts incubated in the presence of 100 μg/ml of CAP. Lanes 3 and 4 are protein from protoplasts incubated in the presence of 1 mM and 100 μM spermine, respectively. All lanes were loaded with equal cpm of [^{35}S]-methionine-labelled product.

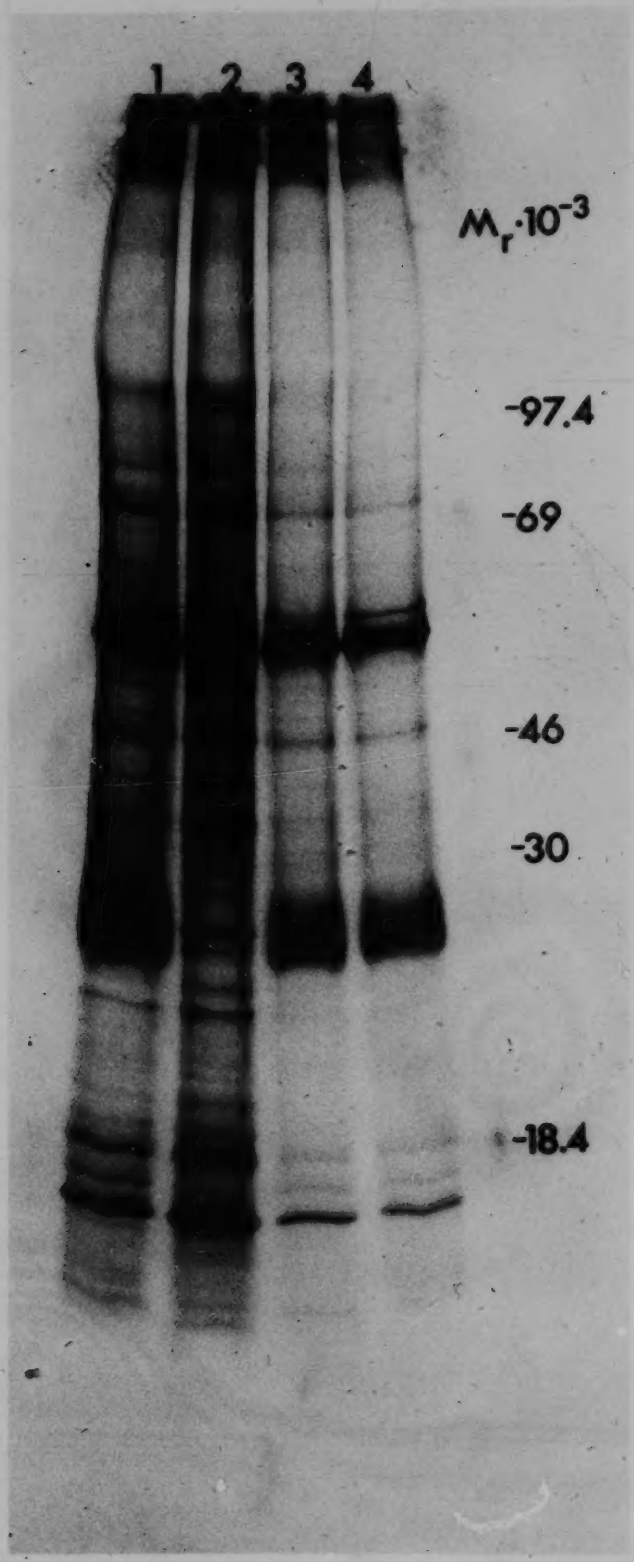

1
2
3
4
$M_r \cdot 10^{-3}$
-97.4
-69
-46
-30
-18.4

Table VIII. Effect of hydrogen peroxide and different spermine preparations on protoplast protein synthesis. Protein synthesis was carried out with 6.5 μCi of [^{35}S]-methionine per 10^4 protoplasts for 60 min under light at 25°C. Values are means of 3 repeats expressed as a percentage of the untreated control. Different protoplast preparations were used for each repeat.

	Treatment	Mean
Spermine, 1 mM	HCl-salt unbuffered *	38a
	HCl-salt buffered with HCl	27ab
	free base unbuffered *	26ab
	HCl-salt buffered with acetic acid	25ab
H_2O_2	50 μM	30ab
	500 μM	7b

* Stock solutions of spermine were unbuffered and the protoplast incubation buffer was increased to 20 mM MES-KOH, pH 5.5.
All means followed by the same letter are not significantly different at the 5% level as determined by LSD.

Another possible explanation for the inhibition of protoplast protein synthesis by polyamines is that polyamine oxidase was rapidly oxidizing the amines to H_2O_2 and aminopropane (112,220). Low concentrations of H_2O_2 were shown to greatly reduce protoplast protein synthesis (Table VIII).

Polyamine oxidase is normally located in the cell wall (112) but the possibility that it was present in the protoplast suspension could not be discounted. A specific inhibitor of polyamine oxidase, β-hydroxyethylhydrazine (112), and the oxygen-radical scavenging enzymes peroxidase and catalase, were used in an attempt to overcome spermine inhibition. These treatments on their own did not affect protein synthesis (Table IX). However, none of the treatments alleviated any of the inhibition experienced with 1 mM spermine (Table IX).

The inhibition of protoplast protein synthesis illustrated in Figure 10 could also be attributed to reduced uptake of radiolabelled methionine. Amine inhibition of betacyanin and ion efflux from beet root discs has been correlated with the degree of amination of the inhibiting compounds (229). The activities associated with plant membrane-bound proteins such as peroxidase (228), ATPase (227) and PET (30) are reported to be inhibited by

Table IX. Influence of a polyamine oxidase inhibitor, catalase and peroxidase on spermine inhibition of protein synthesis. Protoplasts ('700') at 10^6 protoplasts/ml were incubated for 60 min in light at 25°C. Values are means of 3 independent replicates expressed as a percentage of the untreated control. The average amount of [^{35}S]-methionine in TCA-precipitable protein of the control was 253,245 cpm/10^4 protoplasts.

Treatment	Means
β-OH-ethylhydrazine, 50 μM	113a
Peroxidase, 50 μg/ml	105a
Catalase, 50 μg/ml	105a
Spermine, 1 mM	50b
+ Peroxidase	61b
+ Catalase	50b
+ β-OH-ethylhydrazine	46b

All means followed by the same letter are not significantly different at the 5% level as determined by LSD.

polyamines. Gramine is also able to inhibit PET (3). Recently polyamines have been shown to rigidify membranes by interacting with phospholipid head groups (197).

The influence of spermine on methionine and α-aminoisobutryic acid (AIB) uptake by protoplasts was examined using the silicone oil centrifugation method. Methionine uptake was performed in the presence of CAP and CHI so that any possible reduction in the cytosolic pool of methionine as a consequence of protein synthesis would not influence the rate of methionine uptake. Aliquots taken from the protoplast suspensions incubated with [^{35}S]-methionine for 1 h did not contain any TCA-precipitable radioactivity. After 10 min of incubation methionine uptake in the presence of 1 mM spermine had dropped to 80% that of the control rate and by 30 min it was reduced further to 44% (Fig. 13). The control rate was 1230 pmoles μg chl^{-1}h^{-1} (105 pmoles μg protein^{-1}h^{-1}) between 5 and 10 min of incubation. Figure 14 shows that AIB uptake was inhibited to 75% of the untreated control by 10 min and to 52% by 30 min. The rate of AIB uptake by control protoplasts was 80 nmoles μg chl^{-1}h^{-1} (16 nmoles μg protein^{-1}h^{-1}) between 5 and 10 min of incubation. The uptake of AIB by pea protoplasts was reported to be only 0.5 nmoles μg chl^{-1}h^{-1} and the uptake reached a plateau in

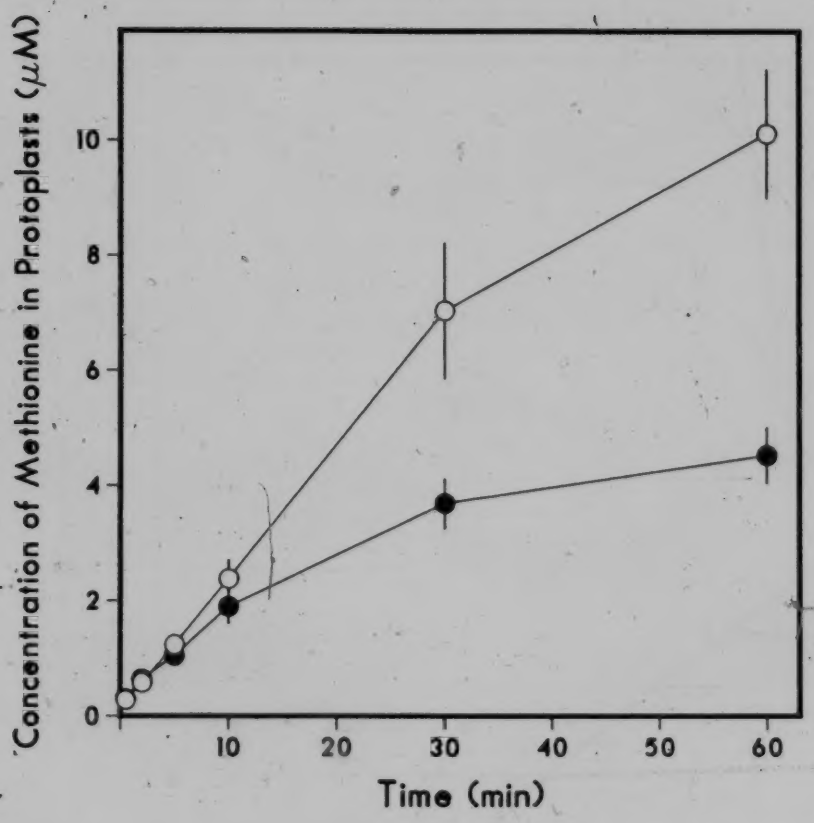


Figure 13. Effect of spermine on the accumulation of methionine by protoplasts. Protoplasts ('700') were incubated with 6 nM methionine, 100 μg/ml CAP and 25 μg CHI in the absence (○) or presence of 1 mM spermine (●). Methionine uptake by protoplasts was evaluated by using silicone oil centrifugation as described in 'Materials and Methods'. Values are means of 3 replicates ± SE.

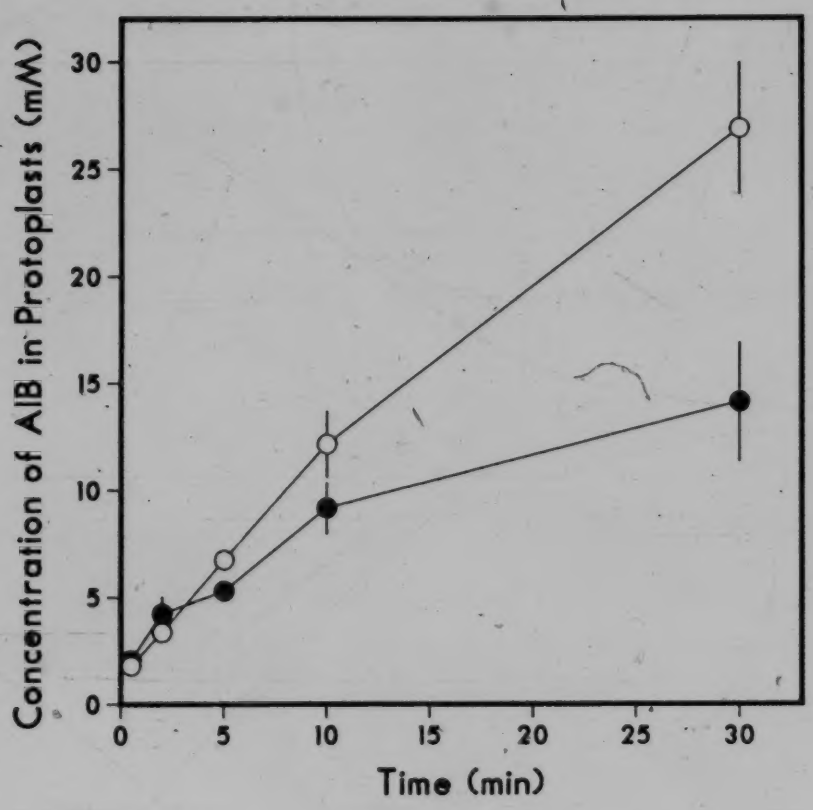


Figure 14. Effect of spermine on the accumulation of AIB by protoplasts. Protoplasts ('700') were incubated with 82 mM AIB in the absence (○) or presence (●) of 1 mM spermine. Uptake of AIB by protoplasts was evaluated by using silicone oil centrifugation as described in 'Materials and Methods'. Values are means of 3 replicates ± SE.

less than 10 min (83), a condition not found with the barley protoplasts. However, soybean sink-leaf protoplasts took up AIB at the rate of 44 nmoles μg protein^{-1}h^{-1} in a linear fashion for at least 30 min (246).

There is considerable evidence to suggest that CAP and CHI exert little or no effect upon amino acid uptake during an incubation period of a few hours (94,178,204,198). However, as assay and protoplast conditions used in this study vary from those used in other reports amino acid uptake was assessed using silicone oil centrifugation. Protoplasts were preincubated with CHI and CAP for 60 min before the addition of AIB. Uptake of AIB was then measured at 30 and 60 min following AIB addition. The amount of AIB taken up at 30 and 60 min by protoplasts treated with CAP and CHI was not significantly different from the untreated control (Table X).

When protoplasts were preincubated with 1 mM spermine for 60 min their subsequent uptake of AIB during a further 30 and 60 min of incubation was inhibited by 34% compared to the untreated control (Table X). The simultaneous addition of AIB and 1 mM spermine (Fig. 14) resulted in 14% more inhibition than observed for protoplasts which were incubated for 60 min with 1 mM spermine before the addition of AIB (Table X). In addition to directly inhibiting the transport of compounds acrpss the plasmalemma, spermine may exert secondary effects which may contribute towards substantial variation in the uptake of AIB. Furthermore, it is not known whether spermine can alter membrane or protein properties such that the silicone oil centrifugation method fails to give a clear separation of intact protoplasts from other material. Further studies are necessary to determine the exact mode by which spermine affects transport across the plasmalemma and whether spermine artifactually affects the silicone oil centrifugation method.

Other researchers have noted that exogenously supplied Ca^{2+} will rigidify plant membranes (128,197). Therefore, it was assumed that Ca^{2+} would inhibit AIB uptake by protoplasts in a manner similar to that of spermine. $CaCl_2$ (10 mM) actually improved the uptake of AIB by almost 100% (Table X). Ca^{2+} has been shown to improve the uptake of

Table X. Effect of protoplast pretreatment with protein synthesis inhibitors, $CaCl_2$ and spermine on AIB accumulation by protoplasts. Protoplasts ('700') at 10^6 protoplasts/ml were preincubated with treatments for 60 min under light at 25°C. Following the 60 min pre-treatment AIB was added to the protoplasts to a final concentration of 82 mM. Thirty and 60 min following the addition of AIB the concentration of AIB (mM) in the protoplasts was determined by silicone oil centrifugation as described in 'Materials and Methods'. Values are means of 3 independent replicates expressed in mM of AIB in the protoplasts.

Treatments	Length of Incubation (min) 30	60	Means
$CaCl_2$, 10mM	40.3	57.8	49.0a
Control	22.5	27.4	25.0b
CAP-CHI*	16.4	23.2	19.8bc
Spermine, 1 mM	15.7	17.4	16.6c
Means	23.7	31.5	
	a	b	

* 100 μg/ml chloramphenicol + 25 μg/ml cycloheximide
The treatment x length of incubation interaction is not significant at the 5% level. Treatments and times of incubation followed by the same letter are not significantly different at the 5% level as determined by LSD.

serine by tobacco cells (221). Smith (221) suggested that Ca^{2+} increased the proton gradient across the tobacco plasmalemma which, in turn, increased the uptake of serine. More recently, AIB, serine and glutamine transport across the plasmalemma was shown to proceed by way of a proton co-transport mechanism (246). Ca^{2+} is capable of stimulating plasmalemma ATPases by binding to calmodulin and subsequently the Ca^{2+}-calmodulin complex stimulates the activity of membrane-bound ATPases (40). It also has been suggested that Ca^{2+} can stimulate ATPases by binding directly to the ATPase protein (40) or by binding to membrane components which can induce conformational changes in the membrane and subsequently an increase in ATPase activity (221). Plasmalemma ATPase activity can generate a proton gradient across the plasmalemma and this proton gradient could be involved in the transport of Ca^{2+} (40), sugars (132,134) and amino acids (246). However, although each of the above steps appears to operate in the plasmalemma, it remains to be shown that Ca^{2+} can stimulate the formation of a proton gradient, which in turn, is responsible for the increase in uptake of amino acids observed in this study and the study by Smith (221).

The inhibition of protoplast protein synthesis by 1 mM spermine varied between 10 and 50% of the untreated control (Fig. 10, Tables VIII,IX). In comparison, amino acid uptake was inhibited approximately 50% by 1 mM spermine (Fig. 13,14 and Table X). From these results it appears that inhibition of protein synthesis by polyamines can be largely attributed to the reduced uptake of [^{35}S]-methionine by the protoplasts. The extent to which polyamines may have adversely affected metabolic processes which are not directly linked to amino acid uptake yet contributed towards the decreased rate of [^{35}S]-methionine incorporation into protein was not assessed. However, polyamines do inhibit the uptake of amino acids and from other studies it is acknowledged that polyamines interfere with the normal function of plant membranes and/or membrane proteins (30,169,196,197,227,228,229). Therefore, the indiscriminate addition of polyamines to protoplasts (and other plant tissues) should be approached with caution.

Light Regulation of Protein Synthesis and Turnover

Protoplasts which were incubated in the dark for 60 min showed some differences both in the rate and the nature of proteins synthesized in comparison to proteins synthesized in the light (Fig. 4, lanes 4 and 7). The amount of TCA-precipitable protein synthesized by protoplasts incubated in the dark was 72±3 (SE) % of that synthesized by protoplasts in the light. From Figure 4 (lane 7) it can be noted that the HBP is not synthesized in the dark to the same extent as in the light yet all other proteins appear to be synthesized equally in the light and in the dark.

Synthesis of the HBP is stringently regulated by light both on the translational (142) and transcriptional level (47,142,254). Degradation of the HBP is also controlled by light and under high light intensities this degradation increases (122,142,173,259). The PET inhibiting herbicides, atrazine and diuron, are able to reduce this rate of degradation (122,173,259). To date, this rapid turnover has been studied in two different plant systems. Initial studies were all performed with aquatic species such as *Spirodela* (95,142) and *Chlamydomonas* (122,259).

As alluded to in the 'Literature Review', the advantage of using these species is that proteins can be rapidly radiolabelled, chemical treatments are taken up rapidly and samples may be easily witdrawn. Thylakoid membranes have been isolated from the radiolabelled plants and *in vitro* treatments which increase or decrease the rate of degradation of the HBP have been imposed on the thylakoid membranes. In order to radiolabel proteins of higher plants, pea shoots were placed in a buffer containing radioactive amino acid. After several hours of incubation the radiolabelled thylakoid membranes were extracted and subjected to *in vitro* treatments similar to those performed on thylakoids from aquatic species (173).

In the present study barley protoplasts were radiolabelled under a light intensity of 350 μE $m^{-2}s^{-1}$ as previously described. The intact, pre-labelled protoplasts were then subjected to various treatments. The change in radioactivity associated with the thylakoid protein extract was determined following the exposure of the protoplasts to the specified light and/or chemical treatments (see legends to Fig. 15 and 16).

The broad band migrating around Mr 30 kD in the gels presented in Figures 15 and 16 is the HBP. The HBP is a very hydrophobic protein (141) and as a consequence it is very difficult to completely denature the HBP with SDS prior to PAGE. The HBP, in different stages of denaturation, appears as a broad band in SDS-polyacrylamide gels (142,143,144,193). More recently, the use of urea as a denaturing agent has improved the denaturation of the HBP and enhanced the sharpness of the HBP band as resolved on polyacrylamide gels (122,173).

The untreated control samples (Fig. 15, lane 7 and Fig. 16, lane 4) are thylakoid membrane proteins extracted from protoplasts immediately following the 60 min preincubation with [^{35}S]-methionine. Each lane of the gels was loaded with thylakoid proteins derived from 1.5 x 10^5 protoplasts.

Under high light intensity (2500 μE $m^{-2}s^{-1}$) the amount of radioactivity associated with the thylakoid proteins of each protoplast sample progressively decreased as a function of length of incubation (Fig. 15, lanes 5,4,3,2, legend). After 60 min of incubation

Figure 15. Fluorograph showing the synthesis and degradation of the HBP in barley protoplasts. Barley protoplasts were initially incubated for 60 min at 1.6 x 10^6 protoplasts/ml with 1.2 mM [^{35}S]-methionine as described in 'Materials and Methods'. Aliquots (each containing 1.5 x 10^5 protoplasts) of the radiolabelled protoplasts were diluted to 10^6 protoplasts/ml with cold methionine (2.5 mM). These samples were incubated at 25°C with the following treatments:
lane 1) cold chase for 60 min in the dark [95]
lane 2,3,4,5) cold chase in light intensity of 2500 μE $m^{-2}s^{-1}$ for 60, 30, 15 and 5 min, respectively [75,81,86,94]
lane 6) cold chase in light intensity of 350 μE $m^{-2}s^{-1}$ for 60 min [78]
lane 7) untreated control [100]
Each lane was loaded with the total thylakoid extract from 1.5 x 10^5 protoplasts. The specific activity of the thylakoid protein from the untreated control sample (lane 7) was 405,000 cpm μg chl^{-1} in the original protoplast sample. Values in square brackets refer to the radioactivity in the thylakoid proteins of the samples loaded in these lanes expressed as a percentage of the untreated control (lane 7).

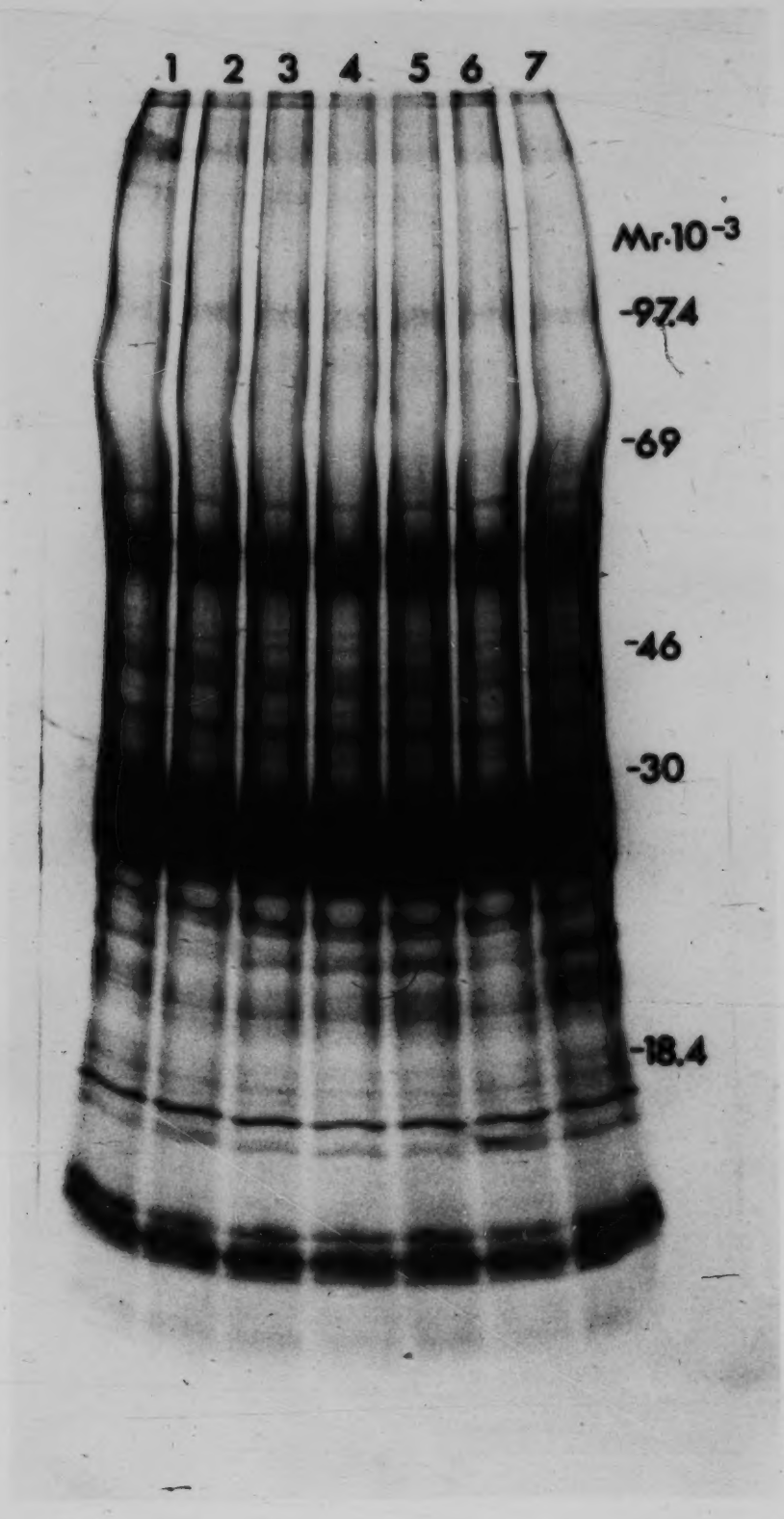
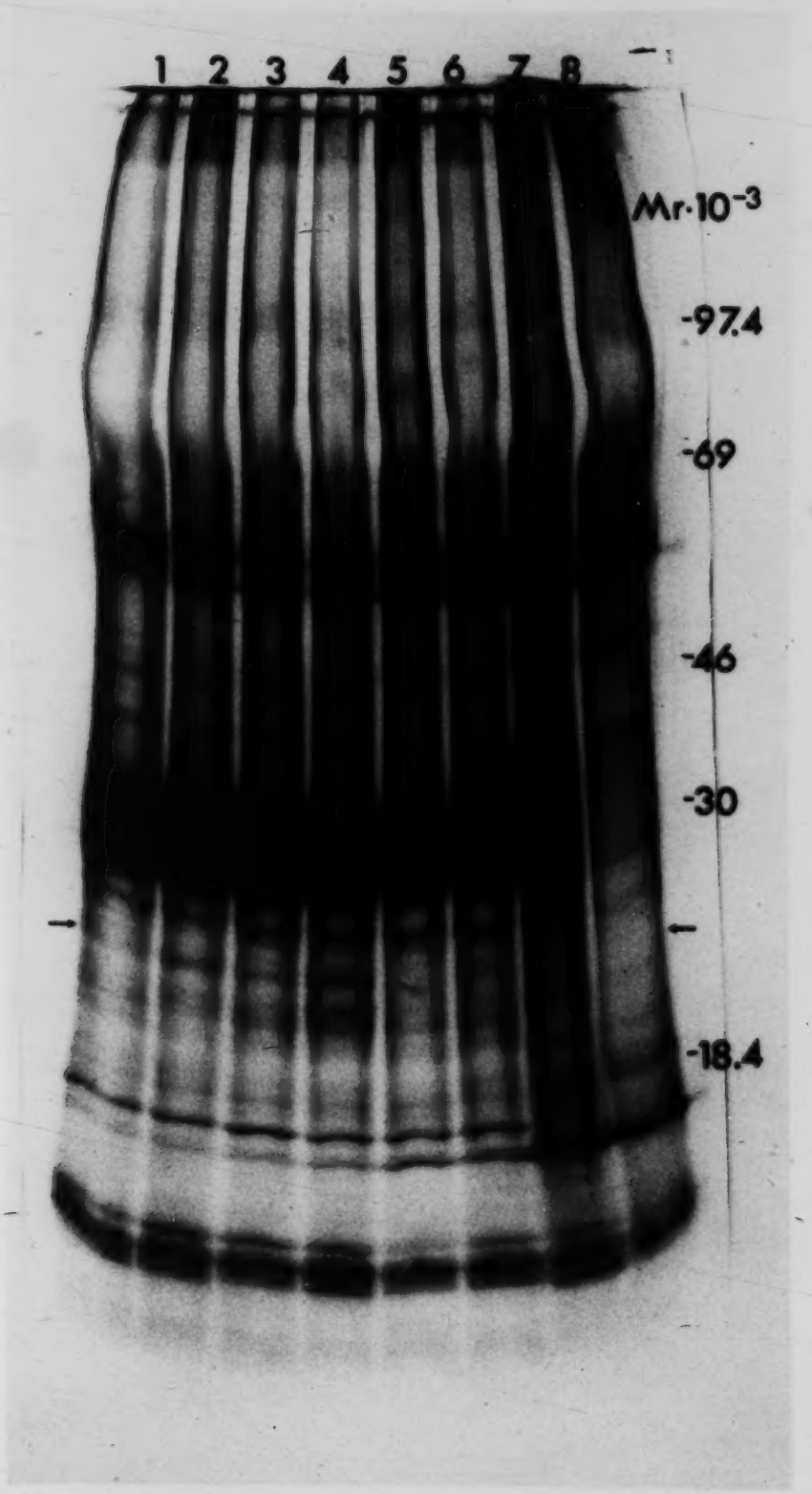

1
2
3
4
5
6
7
Mr·10⁻³
-97.4
-69
-46
-30
-18.4

Figure 16. Fluorograph showing the effect of ADP, diuron, atrazine and spermine on the degradation of the HBP. Barley protoplasts were radiolabelled and incubated as described for Figure 15 and in 'Materials and Methods'. Aliquots were diluted to 10^6 protoplasts/ml with cold methionine (2.5 mM) and the respective chemicals used for treatments. An exception to this was the sample loaded in lane 6 which was diluted with CAP and CHI only. All samples were exposed to light intensity of 2500 $\mu E\ m^{-2}s^{-1}$ for 60 min at 25°C and subjected to the following treatments:

lane 1) 10 mM ADP [84]
lane 2) 10 μM diuron [97]
lane 3) 10 μM atrazine [103]
lane 4) untreated control [100]
lane 5) 10 mM spermine [100]
lane 6) 100 μg CAP + 25 μg CHI [91]
lane 7) 10 mM $HgCl_2$ [167]
lane 8) 10 mM $CuCl_2$ [156]

Each lane was loaded with the total thylakoid extract from 1.5 x 10^5 protoplasts. The specific activity of the untreated control thylakoid protein (lane 4) was 375,000 cpm $\mu g\ chl^{-1}$ in the original protoplast sample. Values in square brackets refer to the radioactivity in the thylakoid proteins of the samples loaded in these lanes expressed as a percentage of the untreated control (lane 4). The same suspension of radiolabelled protoplasts was employed for Figures 15 and 16. The arrow denotes a Mr 29 kD polypeptide which is lost from the thylakoid membranes upon incubation in light.


1 2 3 4 5 6 7 8
Mr·10⁻³
-97.4
-69
-46
-30
-18.4

approximately 20% of the thylakoid protein radioactivity had been lost. The HBP is the prinicipal polypeptide (>50%) which contributes to this decrease in thylakoid protein radioactivity (Fig. 15, lanes 5,4,3,2). In cells of *Spirodela oligorrhiza* the total degradation of HBP required 20 h (142). In comparison, radiolabelled HBP was no longer detectable in *Spirodela* (144), pea (173) or *Chlamydomonas reinhardtii* (259) thylakoid membranes following *in vitro* incubation of the membranes for 60 min. Obviously, the degradation of the thylakoid proteins is accelerated when the stroma and/or cytoplasm is no longer present. Since the catabolism of the HBP does not appear to occur in the dark (Fig. 15, lane 1, 142,173,259), loss of HBP from thylakoid membranes is likely to be photoregulated. Indeed, studies with *Spirodela oligorrhiza* cells (142) and with isolated thylakoid membranes from *Chlamydomonas reinhardtii* (259) have shown that rates of HBP degradation increase upon exposure to higher light intensities. However, in this study the loss of HBP from thylakoid membranes appears to be equally rapid in protoplasts exposed to light intensities of either 350 or 2500 μE $m^{-2}s^{-1}$ (Fig. 15, lanes 2 and 6).

The herbicides atrazine and diuron prevented the degradation of the HBP in the light (Fig. 16, lanes 2,3). These chemicals are known to inhibit PET by binding directly to or near the HBP and thereby changing the conformation of this protein (242). Two explanations can be offered for the effect that these herbicides have on the light-regulated loss of HBP. Firstly, the assumed change in HBP conformation upon binding of herbicide may reduce the susceptibility of HBP to endogenous proteolytic activity. Early studies showed that atrazine and diuron could protect the HBP from cleavage by exogenously added trypsin (144). A second hypothesis concerns the relationship of HBP loss to photoinhibition and the decrease in photosystem II PET activity. In this model high light intensities give rise to maximal rates of PET. These rates of PET are supraoptimal for the photosynthetic membranes and the capacity of the photosystems to transport high energy electrons through the thylakoid membranes is exceeded. It has been proposed that the HBP serves as a scavenger for the electrons lost during PET. In such a role the HBP is sacrificed at the expense of retaining the intactness of

the photosynthetic membranes (6,47,122). The protective role of atrazine and diuron in this model is to displace the quinone from the HBP and therefore prevent the oxidation of the quinone by electrons (6). Recently, this latter hypothesis has been criticized as radical species do not affect the HBP or photosystem II as this hypothesis would assume (161). However, it is still agreed that PET activity is closely related to the degradation of the HBP (161,259).

Roberts *et al.* (196) reported that bean thylakoid membranes treated *in vitro* with spermine did not lose HBP. However, in this study 10 mM spermine did not appear to retard the degradation of HBP (Fig. 16, lane 5). Interestingly, the total radioactivity associated with the thylakoid membranes of barley protoplasts did not change after spermine treatment (Fig. 16, legend). It appears as though spermine treated protoplasts have more LSU associated with the thylakoid pellet which, in turn, has led to the higher estimate for thylakoid radioactivity (compare lane 4 with lanes 2,3 of Fig. 16). The spermine used in this study had to be transported across the plasmalemma and the chloroplast envelope before reaching the thylakoid membrane. Therefore, the actual concentration of spermine to which thylakoid membranes were exposed may have been substantially less than the level used to treat isolated bean thylakoid membranes.

The disulfide bonds in thiolproteases are required for the proteolytic activity of these enzymes (5). Disulfide bonds are reduced by Cu^{2+} and Hg^{2+} and therefore these heavy metals have been used as inhibitors of thiolproteases (5). Wettern and Galling (259) reported that the degradation of HBP could be prevented by the addition of Cu^{2+} and Hg^{2+}. In the present study, when protoplasts were treated with either 10 mM $HgCl_2$ or 10 mM $CuCl_2$ many proteins in addition to the thylakoid polypeptides were precipitated. The presumed 'thylakoid extract' from the the Hg^{2+} and Cu^{2+} treated protoplasts contained greater amounts of contaminating LSU and SSU than found in any of the other thylakoid extracts (Fig. 16, lanes 7,8). Cu^{2+} also had a solubilizing effect on the HBP since this protein appears to have been lost with the supernatant washes and is not present in the 'thylakoid extract' (Fig. 16, lane 8). These findings suggest that the role of thiolproteases in the degradation of HBP

proposed by Wettern and Galling (259) should be reassessed.

Ohad *et al.* (173) reported that 5 mM ADP could reduce the degradation of pea HBP by 92%. ADP can regulate the activity of several kinases which in turn may phosphorylate proteins involved in proteolytic degradation (259). In the present study 10 mM ADP had no effect on HBP loss from the thylakoid membranes (Fig. 16, lane 1). ADP may not have reached the thylakoid membranes or chloroplast stroma in sufficient quantities to exert an effect.

CAP plus CHI can prevent further protein synthesis and therefore CAP plus CHI could be used as a substitute to a cold methionine chase. However, the CAP plus CHI treatment appears to have slightly reduced the degradation of the HBP (Fig. 16, lane 6).

The HBP is not the only protein whose loss/degradation from thylakoid membranes appears to be light-regulated. Holloway *et al.* (96) described six polypeptides (other than HBP) which were progressively lost from wheat thylakoid membranes during exposure to high light intensity. A polypeptide migrating at Mr of 29 kD (indicated by an arrow in Fig. 16) is completely lost from thylakoid membranes during a 60 min incubation in light (Fig. 15, lanes 2,6). The loss of this protein is retarded by diuron and atrazine (Fig. 16, lanes 2,3) and spermine appears to have no effect on the degradation of this protein (Fig. 16, lane 5).

The loss of HBP from thylakoid membranes of barley protoplasts responded to light and herbicide treatments in a manner analogous to that reported for *Spirodela* cells (142) and isolated thylakoid membranes (122,144,173,259). Therefore, protoplasts should be useful as a tool for the study of the synthesis and the degradation of the HBP and other plant proteins. In addition, these photoregulated polypeptides from several different species can be compared simultaneuosly through the use of protoplasts. One limitation to the use of intact protoplasts is the impermeability of the plasmalemma and the chloroplast envelope to various protease inhibitors and other chemicals. However, thylakoid membranes can be isolated from previously radiolabelled protoplasts. These pre-labelled thylakoid membranes could then be used in *in vitro* experiments such as those reported for *Chlamydomonas reinhardtii* and peas.

HBP degradation in protoplasts and in chloroplasts and thylakoid membranes derived from the same isolation of protoplasts might also be a useful comparison. Furthermore, by retaining the chloroplast envelope and/or the plasmalemma the *in vivo* degradation of proteins in the presence of cytosolic and stromal-factors involved in protein synthesis and degradation can be studied.

The use of protoplasts has another major advantage over the shoot labelling technique used for peas (173) and beans (196). The specific activity of the thylakoid membranes used in this study was 400,000 cpm μg chl^{-1}. In comparison, Ohad *et al.* (173) obtained a specific activity of only 10,000 cpm μg chl^{-1}. A higher specific activity enables the detection of HBP breakdown products as well as the improved quantitation of the loss of HBP and other proteins from the thylakoid membranes. In a recent study of HBP degradation, HBP per sé was not quantitated (259). Instead, only the change in radioactivity associated with the total thylakoid pellet was determined. The accuracy of this method of quantitation depends entirely on the purity of the thylakoid extract (see Fig. 16, lanes 5,7,8) and the percentage of radioactivity in the thylakoid extract attributable to HBP. In *Chlamydomonas reinahrdtii* almost 100% of the radioactivity in the thylakoid extract was associated with HBP (259). However, this value is less than 40% for peas (173) and barley (this study). In other studies the radioactivity asssociated with the HBP was evaluated from densitometric scans of fluorographs (173). This method also does not provide an accurate estimate of HBP quantity since the relationship between the radioactivity in the protein and the quantity of silver grains exposed on the film is often not linear especially when there is a large difference in the radioactivity associated with various polypeptides (15). Furthermore, the thickness and density of the gel, gel staining, destaining and drying methods can cause variations in the exposure of film (15). Polyclonal antibodies to the HBP are only available in small quantities (173) and therefore the routine quantitation of HBP through immunoprecipitation is also not possible. In summary, there is a need for improved methods for the quantitation and detailed biochemical study of this protein.

V. Conclusions

In 1967 Otsuki and Takebe (176) isolated mesophyll cells and protoplasts from a number of plant species. They determined that the optimal concentration of osmoticum for most of the species tested was 800 mM (176,234) and noted that protoplasts suspended in 300 mM mannitol were easily ruptured (234). Results from the present study using barley protoplasts also indicate that plasmalemma intactness is greatest when protoplasts are suspended in buffers of a high osmotic concentration (Fig. 1, Table II). Barley protoplasts suspended in 400 or 500 mM sorbitol are typically swollen and tend to lyse more readily than the slightly plasmolyzed protoplasts suspended in 700 mM sorbitol (Fig. 1). Several studies completed since the initial work by Takebe and co-workers have also found that buffers of high osmotic concentration result both in improved rates of protoplast survival (67,167,174,209,266) and the improved regeneration of plants from protoplasts (118,225).

While protoplasts suspended in buffers of high osmotic concentration may survive longer there are conflicting reports on the relationship between a number of protoplast metabolic events and osmotic tension. RNase activity in tobacco and oat protoplasts increases rapidly following isolation. Indeed, 48 h after isolation RNase activity is 10 to 20 times higher than observed immediately after protoplast isolation (67,126,186). This dramatic increase is believed to be the result of *de novo* synthesis of RNase (186). Farkas and co-workers also noted that this rise in RNase activity was greater for hypertonic protoplasts than for hypotonic protoplasts (126,186). They concluded that high osmotic stress induced the increase in protoplast RNase. However, if osmotic stress is responsible for the increase in RNase activity in protoplasts then protoplasts suspended in a low osmotic concentration would be expected to give rise to only modest increases in RNase activity. These same authors (186) reported that RNase activity in hypertonically held tobacco protoplasts (400 mM mannitol) increased 13-fold within 48 h while RNase activity in protoplasts suspended in 700 mM mannitol increased 18-fold within the same time period. These observations would suggest that the induction of *de novo* synthesis of RNase appears to occur in protoplasts subjected to

both low and high osmotic concentrations. The slightly higher level of RNase activity in the 48 h old, hypertonic protoplasts may simply be indicative of superior rates of protein synthesis in the hypertonically held protoplasts as was noted for barley (Table V). Unfortunately, Farkas and co-workers have presented somewhat contradictory results concerning the effects of osmotic concentration on the protein synthesizing capabilities of tobacco protoplasts (187). In conclusion, the observed increases in RNase activity in tobacco protoplasts may not be induced by 'osmotic stress'. The distinctive property of tobacco and oat protoplasts which leads to the increases in RNase levels remains elusive. In this study RNase activity had not increased in the barley protoplasts during a 30 h incubation period (Table III). This is not a particularily surprising observation as the barley protoplasts did not survive for extended periods (Table II) which presumedly prevented *de novo* synthesis of RNase. However, the aged '500' protoplasts did exhibit slightly higher RNase activity than similarily aged '700' protoplasts (Table III) even though the '700' protoplasts possessed a greater capacity for protein synthesis.

Table I illustrates that the carboxylation reactions of photosynthesis were more efficient in '700' as opposed to '500' barley protoplasts. In contrast, hypotonically held spinach protoplasts were more photosynthetically competent than hypertonically held protoplasts (108). As discussed previously in 'Results and Discussion' the interpretation of the data of Kaiser *et al.* (108,107) and of this study (Table I) with regards to photosynthesis and osmotic concentration are conflicting. Further experimentation is required in order to resolve the discrepancies in these results.

A few papers have also reported that protoplast protein synthesis is adversely affected by high osmotic concentration (58,187). Premecz *et al.* (187) do not present the data which they claim shows histidine incorporation into protein is higher for tobacco protoplasts suspended in 400 versus 700 mM mannitol. They do, however, present data which illustrates that the quantity of TMV protein synthesized by protoplasts exposed to different osmotic concentrations is equal (187). Fleck and co-workers (58) also claim that osmotic stress

reduces the rate of protoplast protein synthesis. A criticism that may be raised against this study is that the authors drew their conclusions entirely from comparisons made between cells, severed leaves and protoplasts. They did not compare protoplasts held at different osmotic concentrations. Table V shows that of barley protoplasts incubated in a range of four osmotic concentrations, protoplasts suspended in 700 mM sorbitol had higher rates of protein synthesis. Therefore, protoplast protein synthesis does not seem to be adversely affected by high osmotic concentration as originally claimed (58,187).

Barley protoplast protein synthesis was greatly reduced when protoplasts were suspended in buffers of low osmotic concentration (Table V). Barley protoplasts suspended in 400 mM sorbitol tended to lyse easily (Fig. 1) and reduced protoplast intactness may, at least in part, explain the reduced rates of protein synthesis found for '400' and '500' protoplasts (Table V). Effects of low osmotic concentration on protoplast protein synthesis could not be explained by differences in RNase activity (Tables II,III) or mRNA quantities (Fig. 9). Other factors which may vary with osmotic concentration and subsequently affect the apparent rate of protein synthesis include uptake of radiolabelled amino acids and/or changes in proteolytic activity. These conditions were not examined in this study.

Fleck and co-workers (58) suggested that the proteins synthesized by protoplasts were qualitatively different from those synthesized *in vivo* due to osmotic stress. However, in the current study barley protoplasts suspended in 400, 500, 600 or 700 mM sorbitol synthesized the same complement of abundant polypeptides (Fig. 4,5). Also, the fraction of proteins contributed by the chloroplasts or the cytoplasm of barley protoplasts did not vary with osmotic concentration (Table V, Fig. 4). Therefore, any major differences in the proteins synthesized by protoplasts in comparison to leaves are not likely to be the result of osmotic pressure.

A few studies have indicated that there are numerous differences in the proteins synthesized by protoplasts, cells and severed leaves (57,58). The SSU of Rubisco is reported as both absent (59,57) and present (58,94) among products labelled by tobacco protoplasts. In

the present study barley protoplasts appeared to synthesize less SSU than did severed barley leaves (Fig. 8). In efforts to explain the reduced quantities of SSU in protoplasts the mRNA coding for the SSU has been quantitated in tobacco and barley protoplasts. The pSSU was absent among wheat germ *in vitro* translation products directed by poly(A) RNA isolated from tobacco protoplasts (57,58). Furthermore, the use of a specific cDNA probe for pSSU mRNA failed to detect any message coding for this polypeptide among the poly(A) RNA fraction of tobacco protoplasts (245). However, in this study readily detectable levels of pSSU mRNA could be demonstrated using dot-blot hybridizations of barley protoplast total RNA (Fig. 9). Two explanations for these divergent observations can be entertained. One possibility is that the conditions under which tobacco protoplasts were examined by Fleck *et al.* (57,58) resulted in either reduced transcription and/or enhanced degradation of pSSU mRNA. Alternatively, as the fraction of tobacco RNA examined was poly(A) RNA, the extent of polyadenylation may influence the apparent recovery and subsequent quantitative estimate of pSSU transcripts. Since the total RNA fraction of barley protoplasts did contain detectable levels of pSSU mRNA in future it may be judicious to use total RNA as the source for transcript quantitation. To date little is known about the purpose of polyadenylation (19). If mRNA polyadenylation does actually decrease rapidly in protoplasts, protoplasts may prove to be a useful tool for the study of polyadenylation.

In addition to SSU, the barley protoplasts synthesized a variety of other polypeptides, but not nearly as many as were labelled by severed barley leaves. However, the HBP was synthesized in larger amounts by the protoplasts than by the severed leaves (Fig. 8). As discussed in 'Results and Discussion' the complement of polypeptides synthesized by leaf tissue *in vivo* is not necessarily identical to that synthesized by severed leaves. Indeed, since the array of proteins synthesized by protoplasts and aquatic plant species are fairly similar, protein synthesis in protoplasts may be more representative of *in vivo* leaf protein synthesis. The exact conditions inherent to protoplasts and severed leaves which leads to their differential synthesis of polypeptides is not known. Of additional interest are the differences

between proteins synthesized by tobacco and barley protoplasts. Future investigations into these discrepancies may assist in improving the viability and subsequent regeneration of cereal protoplasts.

Researchers attempting to improve the survival of protoplasts, particularly those from Gramineae species, have been met with an inordinate number of problems (183,243). Barley protoplasts in this study did not survive for longer than 72 h and 50% of the protoplasts were no longer viable after 18 h (Table II). The survival of protoplasts suspended in buffer of higher osmotic concentration (700 mM sorbitol) was only slightly better than those suspended at a lower osmotic concentration. The addition of 1 mM spermine to protoplasts only marginally improved the survival of protoplasts (Table II). Although decreased protoplast survival may be related to decreased rates of protein synthesis, it may also be a function of a nutrient deficiency or the accumulation of toxic wastes. The above circumstances, including the protein synthetic capacity of the protoplasts in relation to their age, were not fully addressed in this study or in previous studies conducted with Gramineae protoplasts.

Barley protoplasts isolated and incubated in 700 mM sorbitol synthesized protein in a linear fashion for at least 60 min at the rate of 7 fmoles $min^{-1}\mu g\ chl^{-1}$. Chloroplasts in the barley protoplasts synthesized protein at rates equivalent to the highest reported rate for *in organello* protein synthesis (Table V, 175). Furthermore, thylakoid polypeptides of barley protoplasts were labelled to a specific activity 20 times greater than that reported for thylakoids isolated from radiolabelled severed pea shoots (173). CAP and CHI proved to be rapid and relatively specific inhibitors of protein synthesis in protoplasts. These chemicals did not significantly alter amino acid uptake (Table X). Cations, betaine and low concentrations of polyamines did not affect protoplast protein synthesis significantly (Table VI,VII, Fig.10). High pH, DCCD (Table VII), acetate (Tables VI,VIII) and 1 mM concentrations of polyamines (Fig. 10) inhibited the incorporation of methionine into protein. The immediate effect of these chemical treatments may not be at the level of protein synthesis. Moreover, the chemicals may be interfering with the transport of the [^{35}S]-methionine across the

plasmalemma. Spermine (1 mM) did inhibit the uptake of both AIB and methionine by protoplasts (Fig. 13,14, Table X). In contrast, the uptake of AIB was increased twofold by the presence of 10 mM $CaCl_2$ (Table X) yet, $CaCl_2$ did not improve the incorporation of methionine into protein (Table VI). The silicone oil centrifugation technique of Heldt (90) proved to be a rapid, reliable and reproducible method for the measurement of amino acid uptake. In addition, this method provided absolute values on the rate of uptake and the internal concentration of the assayed compound.

There is a rather extensive list of publications which extoll the virtues of polyamines for the improvement of protoplast stability (67,109) and which draw attention to the positive correlation between polyamine titer in plant tissue and plant metabolism and growth (55,114,177). Consequently, the negative effect of polyamines on amino acid uptake was not expected. However, the results illustrated in Figures 13 and 14 and Table X are not the first reports which suggest that polyamines inhibit metabolite transport across the plasmalemma. Srivastava and Smith (229) showed that various polyamines reduce membrane permeability to ions and betacyanin. However, the transport of some compounds across the plasmalemma does not appear to be adversely affected by polyamines. For example, the polyamine-stimulated increase in uridine and thymidine incorporation into nucleic acids (111,220) suggests that the transport of these bases was not reduced by polyamines. Additional effects of polyamines on membrane function were obtained by Roberts *et al.* (197). These authors found that polyamines increased the rigidity of the membranes by specifically reducing the vibration of phospholipid head groups. An alteration in membrane fluidity may in turn be indicative of reduced membrane permeability and explain the reduced activity of membrane peroxidase (228), ATPase (227) and PET (182). A reduction in the rate of the turnover of the membrane-bound HBP by spermine has also been reported (196). However, the same effect of spermine on HBP turnover in thylakoids of barley protoplasts could not be demonstrated (Fig. 16).

Protoplasts have been used to investigate many aspects of plant metabolism but they have been used only sparingly for the study of protein synthesis and degradation. Protein synthesis in barley protoplasts appears to respond to a variety of treatments in manners analagous to those reported for leaf tissue and aquatic plant cells. For example, proteins which are stringently regulated by light (eg. HBP) were synthesized in reduced quantities in the dark (Fig. 4). Furthermore, the degradation of HBP in barley protoplasts was stimulated by light and retarded by the PET inhibiting herbicides, diuron and atrazine, as it is in aquatic cells (142) and isolated thylakoid membranes (173,259).

Despite the above mentioned qualities which suggest that protoplasts could be advantageous systems for research in plant metabolism, a number of properties are discouraging to the future use of protoplasts as biochemical tools. These unfavourable characteristics are their poor survival rate (especially for Gramineae), the unexplained and somewhat variable increases in RNase and the rather perturbing question of exactly how closely protoplast metabolism reflects *in vivo* anabolic and catabolic processes. These unresolved questions are hindrances to maximizing the use of protoplasts in biochemical studies and limit their potential application in plant breeding. This investigation into barley protoplast metabolism has shown that protoplasts are useful in the study of plant biochemical questions. However, researchers, must excercise caution in the interpretation of protoplast-derived results as numerous properties unique to protoplasts are only poorly understood and furthermore problems inherent to their use still need to be overcome.

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